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# Reviews of Physiology, Biochemistry and Pharmacology 174

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# Ezrin Orchestrates Signal Transduction in Airway Cells



Lei-Miao Yin, Ting-Ting Duan, Luis Ulloa, and Yong-Qing Yang

**Abstract** Ezrin is a critical structural protein that organizes receptor complexes and orchestrates their signal transduction. In this study, we review the ezrin-mediated regulation of critical receptor complexes, including the epidermal growth factor receptor (EGFR), CD44, vascular cell adhesion molecule (VCAM), and the deleted in colorectal cancer (DCC) receptor. We also analyze the ezrin-mediated regulation of critical pathways associated with asthma, such as the RhoA, Rho-associated protein kinase (ROCK), and protein kinase A (cAMP/PKA) pathways. Mounting evidence suggests that ezrin plays a role in controlling airway cell function and potentially contributes to respiratory diseases. Ezrin can participate in asthma pathogenesis by affecting bronchial epithelium repair, T lymphocyte regulation, and the contraction of the airway smooth muscle cells. These studies provide new insights for the design of novel therapeutic strategies for asthma treatment.

**Keywords** Actin-binding proteins • Airway cells • Asthma • Ezrin

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## 1 Introduction

Asthma is a clinical challenge in modern medicine that affects over 300 million people and causes over 250,000 deaths annually worldwide (D'Amato et al. 2016; Lambrecht and Hammad 2015). Structural airway cells, such as smooth muscle and epithelial cells, are critical factors that contribute to asthma. Multiple studies suggest that alterations in the actin cytoskeleton cause a pathological contraction of the structural airway cells, which contributes to asthma (Fletcher and Mullins 2010; Noble et al. 2014). In clinical studies, biopsied tissue from asthmatic patients showed reduced  $\beta$ -actin mRNA levels (Glare et al. 2002). Altogether, these results encouraged investigators to study the actin-binding proteins in the airway cells and their clinical implications in asthma (Tang 2015).

This review analyzes recent studies on ezrin and its potential implications in asthma. Ezrin is a principal member of the ERM (ezrin–radixin–moesin) protein family, which includes actin-binding proteins of the band 4.1 superfamily because their N-termini are similar to those of the erythrocyte cytoskeletal protein band 4.1 (Sagara et al. 1995; Vaheri et al. 1997; Gould et al. 1989; Ng et al. 2001). The ERM proteins are known as structural organizers that link membrane proteins to the underlying actin cytoskeleton. In addition to their structural role, the ERMs also regulate the interaction between receptor complexes and intracellular proteins, thereby modulating signal transduction pathways, such as the RhoA, Rho-associated protein kinase (ROCK), and cyclic AMP/protein kinase A (cAMP/PKA) pathways (Iontcheva et al. 2004; Ponta et al. 2003; Celik et al. 2015). The ERMs are expressed in a developmental and tissue-specific pattern. Ezrin is mainly expressed in lymphocytes and epithelial cells, moesin is expressed in endothelial cells, and radixin is expressed in hepatocytes. Ezrin has recently attracted the



attention of multiple investigators due to its role in key biological processes such as the immunological synapsis in T lymphocytes and the epidermal growth factor (EGF)-induced stimulation of human carcinoma tumor differentiation and metastasis (Bretscher et al. 1997; Yoshida et al. 2016). The interaction between ezrin and various receptor complexes and intracellular targets is mainly regulated by phosphorylation (Neisch and Fehon 2011). In this study, we review recent results of ezrin regulation and its physiological and clinical implications.

## 2 Molecular Features of Ezrin

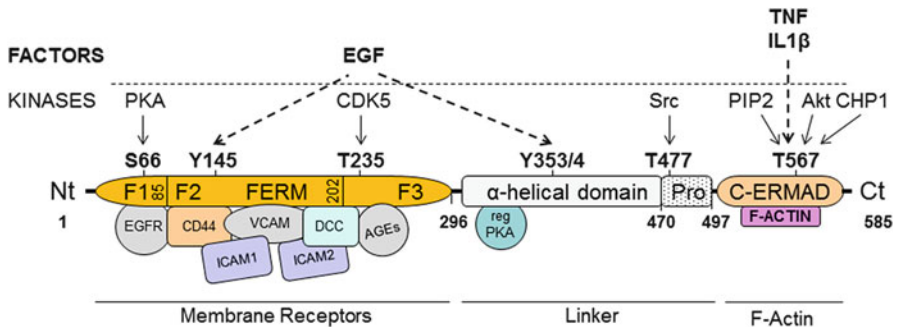
### 2.1 *Discovery of Ezrin*

Ezrin was discovered in multiple cellular processes and was thought to be different proteins because of its different electrophoretic mobility on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Gould et al. 1989; Chambers and Bretscher 2005). Ezrin was first identified in 1981 as a polypeptide with an apparent molecular weight of 81-kDa on SDS-PAGE that was quickly phosphorylated after the stimulation of *human* A431 carcinoma cells with EGF (Hunter and Cooper 1981). In 1983, ezrin was purified as a polypeptide with an apparent molecular weight of 80-kDa on SDS-PAGE from the microvillus cytoskeleton in *chicken* intestinal epithelial cells (Bretscher 1983). In 1986, both polypeptides were compared and identified as ezrin (true molecular weight of 69-kDa), which was confirmed by immunoblotting, immunoprecipitation, two-dimensional gel electrophoresis, and protein sequencing (Gould et al. 1986). Ezrin was also purified as an 82-kDa tumor antigen from the cytosol of a methylcholanthrene-induced sarcoma (Ullrich et al. 1986; Fazioli et al. 1993). In 1988, ezrin was isolated from the microvillar membranes of *human* choriocarcinoma cells as a 75-kDa protein named cytovillin (Pakkanen et al. 1987; Turunen et al. 1989). Ezrin was also purified as a 78-kDa cyclic AMP-dependent kinase anchoring protein (originally named AKAP78) enriched in *murine* gastric parietal cells (Dransfield et al. 1997). These studies showed that ezrin is involved in multiple cellular processes ranging from the EGF stimulation of *human* carcinoma cells to tumor antigens. These results also indicated that the original confusion was due to the different electrophoretic mobility of ezrin on SDS-PAGE (Gould et al. 1989). These differences were mostly due to its phosphorylation, indicating that ezrin is regulated by phosphorylation, which induces structural changes that affect its electrophoretic mobility.

## 2.2 Ezrin Phosphorylation

Ezrin contains an N-terminal FERM (four-point one, ezrin, radixin, moesin) domain (~300 residues), a central linker region (~200 residues), and a C-terminal ERM-associated domain (C-ERMAD, ~80 residues; Fig. 1) (Jayasundar et al. 2012). The N-terminal FERM domain consists of the following three subdomains: F1–F3. These subdomains have structural (but not sequence) homology to known folded proteins. F1 is similar to ubiquitin, F2 is similar to acyl-CoA-binding protein, and F3 is similar to the PTB (phosphotyrosine binding)-domain. All ERMs have a central helical linker region composed of the predicted  $\alpha$ -helical domain. Ezrin and radixin, but not moesin, have a proline-rich linker domain (~470–497 residues) preceding the C-ERMAD. Ezrin is normally a dormant inactive protein due to the intramolecular interaction between the N- and C-terminal domains. Ezrin is activated by phosphorylation, which dissociates the intramolecular interaction between the N- and C-terminal domains and allows the N-terminal domain to interact with membrane receptor complexes and the C-terminal domain to interact with F-actin (Bretscher et al. 1997). Thus, the interaction between ezrin and other proteins is regulated by its phosphorylation in multiple domains by various kinases (McRobert et al. 2003; Fehon et al. 2010). A detailed list of the ezrin phosphorylation sites, kinases, and biological activity is provided in Table 1.

The most common ezrin phosphorylation site is threonine-567 in the C-terminal domain (Zhu et al. 2007). Ezrin threonine-567 is phosphorylated by calcineurin homologous protein-1 (CHP1), and the inhibition of CHP1 abrogates the interaction between ezrin and the  $\text{Na}^+/\text{H}^+$  exchanger 3 (Di Sole et al. 2009). The phosphorylation



**Fig. 1** Structural characteristics of ezrin. Ezrin contains an N-terminal FERM domain (~300 residues), a central linker region (~200 residues), and a C-terminal ERM-associated (C-ERMAD) domain. The N-terminal domain binds to membrane receptor complexes. The linker is an  $\alpha$ -helix with a proline-rich domain. The C-terminal C-ERMAD domain binds to F-actin. Ezrin is regulated by phosphorylation at serine-66, tyrosine-145, threonine-235, tyrosine-353, threonine-477, and threonine-567. These phosphorylations are regulated by multiple extracellular factors (EGF, TNF, and IL1 $\beta$ ) and intracellular kinases (PKA, CDK5, Src, and Akt). FERM four-point one, ezrin, radixin, moesin, EGF epidermal growth factor, TNF tumor necrosis factor, IL1 $\beta$  interleukin-1 $\beta$ , PKA protein kinase A, CDK5 cyclin-dependent kinase 5, SRC proto-oncogene tyrosine-protein kinase Src, PIP<sub>2</sub> phosphatidylinositol 4,5-bisphosphate, CHP1 calcineurin homologous protein-1

**Table 1** Phosphorylation sites for the ezrin activation

Phosphorylation site	Kinases/ phosphorylation factors	Cell line	Biological activity	References
Threonine-567	Phosphatidylinositol 4,5-bisphosphate (PIP <sub>2</sub> )	LLC-PK1 epithelial cell line	Epithelial cell morphogenesis	Fievet et al. (2004)
Threonine-567	Calyculin A	LLC-PK1 epithelial cell line	Cytoskeleton arrangement and development of multicellular epithelial structures	Gautreau et al. (2000)
Threonine-567	Calcineurin homologous protein-1	Opossum kidney cells	Na <sup>+</sup> transport	Di Sole et al. (2009)
Threonine-567	TNF- $\alpha$ and IL-1 $\beta$	Human synoviocytes	Migration and invasion of fibroblast-like synoviocytes	Xiao et al. (2014)
Threonine-567	Akt	BeWo trophoblastic cells from human choriocarcinoma	Microvilli-mediated mechanoresponsive cellular functions, such as epithelial absorption, signal perception, and mechanotransduction	Miura et al. (2015)
Tyrosine-353	Akt (but not ERK1/2, ROCK1) pathway	Tongue squamous cell carcinomas (TSCC) cell	Metastasis of TSCC cells	Wang et al. (2014)
Threonine-567/ Tyrosine-353	N/A	Pancreas tissue	Associated with positive lymph node metastasis, less differentiation, pAkt overexpression, and shorter survival times	Cui et al. (2010)
Threonine-567/ Tyrosine-353	N/A	Tissue of intraductal papillary mucinous neoplasms (IPMNs) and pancreatic intraepithelial neoplasia (PanINs)	Associated with tumor invasion and related to early development of PanINs	Oda et al. (2013)
Tyrosine-145/ Tyrosine-353	EGFR (epidermal growth factor receptor)	Human epidermoid carcinoma A431 cell	Unclear	Krieg and Hunter (1992)

(continued)

Table 1 (continued)

Phosphorylation site	Kinases/ phosphorylation factors	Cell line	Biological activity	References
Threonine-235	CDK5 (cyclin-dependent kinase 5)	Human osteosarcoma cell line SAOS-2	pRb activity and cytoskeletal regulation	Yang and Hinds (2003)
Threonine-235	CDK5	Human osteosarcoma cell line SAOS-2, senescent human diploid fibroblasts	Prevent senescence-associated flat cell formation	Yang and Hinds (2006)
Serine-66	PKA	Gastric parietal cells	Remodeling of the apical membrane cytoskeleton associated with acid secretion	Zhou et al. (2003)
Threonine-477	Src	Human embryonic kidney 293 cells	Unclear	Heiska and Carpen (2005)

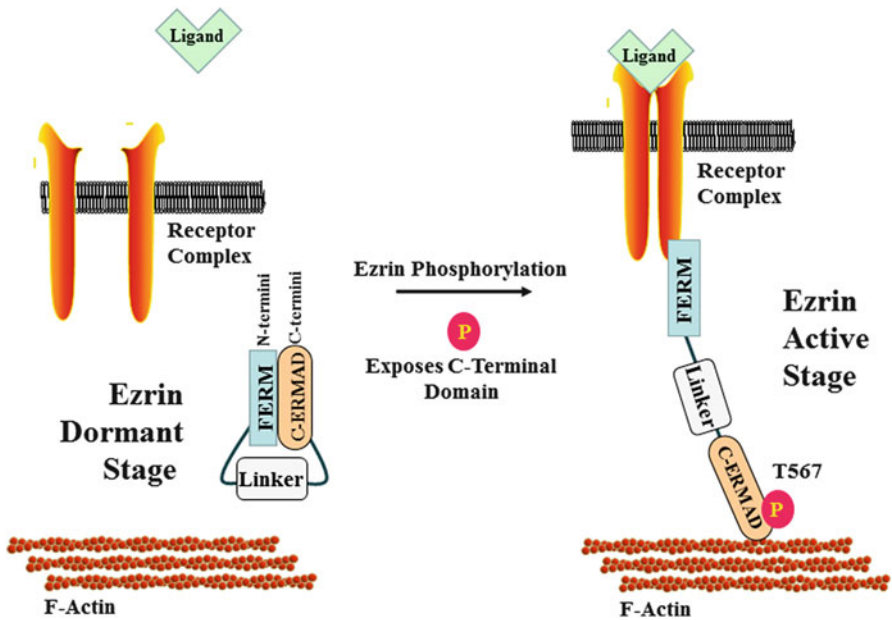
of threonine-567 via phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) is necessary for ezrin activation during kidney epithelial LLC-PK1 cell morphogenesis (Fievet et al. 2004). Ezrin threonine-567 is also phosphorylated by Akt, which induces microvilli in *human* BeWo trophoblastic cells (Miura et al. 2015). The phosphorylation of threonine-567 enhances the binding of ezrin to the actin cytoskeleton and is crucial for establishing epithelial polarity in epithelial LLC-PK1 cells (Gautreau et al. 2000). The phosphorylation of ezrin threonine-567 contributes to the migration and invasion of fibroblast-like synoviocytes in rheumatoid arthritis (Xiao et al. 2014). The ezrin C-terminal domain is also regulated by the phosphorylation of threonine-477. The inhibition of *Src* by the PP2 chemical inhibitor prevented the phosphorylation of ezrin threonine-477 that is induced by pervanadate in *human* embryonic kidney 293 cells (Heiska and Carpen 2005). The phosphorylation of threonine-477 by *Src* enhances the binding of ezrin to the Kelch-repeat and BTB/POZ domain containing 2 (KBTBD2) complex, which suggests that ezrin regulates cellular morphology and adhesion (Heiska and Carpen 2005).

The phosphorylation of the N-terminal domain regulates the binding of ezrin to a variety of proteins and membrane complexes. PKA phosphorylates ezrin serine-66 during the remodeling of the apical membrane during the acid secretion of gastric parietal cells (Zhou et al. 2003). CDK5 (cyclin-dependent kinase 5) induces the phosphorylation of ezrin threonine-235, which regulates the cellular shape and cytoskeletal activity in *human* osteosarcoma SAOS-2 cells (Yang and Hinds 2003). The phosphorylation of threonine-235 by CDK5 causes ezrin to dissociate from the Rho GDP-inhibitor (Rho-GDI) and prevents senescence-associated flat cell formation in SAOS-2 cells and *human* diploid fibroblasts (Yang and Hinds 2006).

Ezrin is also regulated by tyrosine phosphorylation in both the N-terminal domain (Y145) and the helical linker (Y353/4) (Fehon et al. 2010). No tyrosine phosphorylation has been reported in the C-terminal domain, suggesting that tyrosine phosphorylation does not regulate the binding of the ezrin C-terminal domain to F-actin (Fehon et al. 2010). As mentioned above, ezrin was originally identified as a protein that was quickly phosphorylated at tyrosine-353 after the stimulation of *human* epidermoid carcinoma A431 cells with EGF (Hunter and Cooper 1981; Krieg and Hunter 1992). Ezrin is phosphorylated at both tyrosine-145 and 353 during carcinoma differentiation and invasion (Saygideger-Kont et al. 2016; Bretscher 1989). The phosphorylation of ezrin tyrosine-353 is related to tumor differentiation associated with positive lymph node metastasis and shorter survival times in *human* invasive pancreatic carcinomas (Wang et al. 2014; Fehon et al. 2010; Cui et al. 2010). The phosphorylation of ezrin tyrosine-353 by Akt (but not ERK1/2 or ROCK1) has been associated with the metastasis of tongue squamous cell carcinomas (Wang et al. 2014). This phosphorylation induced invasive ductal carcinoma in *human* pancreatic intraepithelial neoplasia (Oda et al. 2013). These results indicate that ezrin is regulated by multiple kinases (PKA, Akt, and *Src*) during critical cellular processes including carcinoma tumor differentiation, survival, and metastasis.

### 3 Ezrin Counterparts: From Receptors to Scaffold Proteins

Ezrin is considered a key regulator of airway cells that modulates the membrane receptor complexes and their signal transduction pathways (Neisch and Fehon 2011; Miura et al. 2015; Perez-Cornejo et al. 2012; Fievet et al. 2007). Ezrin is expressed in airway cells, including both epithelial and smooth muscle cells, and interacts with receptor complexes via its N-terminal domain and the F-actin cytoskeleton via its C-terminal domain (Miura et al. 2015). A schematic of the interaction between ezrin and the membrane receptor complexes is shown in Fig. 2. These interactions are critical for modulating receptor localization, complex organization, and signal transduction pathways. Ezrin regulates critical protein including epidermal growth factor receptor (EGFR), CD44, vascular cell adhesion molecule (VCAM), and deleted in colorectal cancer (DCC) receptor.



**Fig. 2** Structural regulation of ezrin. Ezrin is normally in a dormant inactive stage with its N-terminal domain interacting and blocking the C-terminal domain. Ezrin is activated by phosphorylation. Ezrin threonine-567 is one of the most characteristic phosphorylation sites, and phosphorylation at threonine-567 causes the dissociation of the intramolecular interaction between the N- and C-terminal domains. This dissociation allows the N-terminal domain to interact with multiple receptor complexes and the C-terminal domain to interact with F-actin. *P* phosphorylation

### 3.1 EGFR

Ezrin is phosphorylated at tyrosine-145 in the N-terminus and tyrosine-353 in the central helical domain after the stimulation of *human* A431 epidermoid carcinoma cells with EGF (Hunter and Cooper 1981). These phosphorylations are associated with cellular differentiation and invasion (Saygideger-Kont et al. 2016; Bretscher 1989). Recent studies have shown that ezrin colocalized with EGFR, Na<sup>+</sup>/H<sup>+</sup> exchanger 1 (NHE1), and  $\beta_1$ -integrin during invadopodia formation in tumor invasion and metastasis (Antelmi et al. 2013). The airway epithelial cells express high levels of EGFR during immune responses and cell remodeling in asthma and smoking. These results are consistent with multiple studies showing the potential regulation of EGFR signaling by ezrin (Ammar and Alice 2016; Jing et al. 2015; Burgel and Nadel 2008; Koff et al. 2008; Homma et al. 2015). The EGFR is activated by endothelin-1 in asthmatic airway smooth muscle cells and is involved in airway remodeling in asthma (Ammar and Alice 2016). EGFR also mediates the smoking-induced airway epithelium remodeling (Jing et al. 2015). These results suggest that ezrin contributes to the EGFR-induced modulation of airway cell remodeling and, thereby, respiratory disorders, such as asthma.

### 3.2 CD44 Receptor

Ezrin can also contribute to asthma by inducing CD44 de-polymerization (Lackie et al. 1997; Klagas et al. 2009; Casalino-Matsuda et al. 2009). CD44 is a trans-membrane glycoprotein that is highly expressed on the surface of both immune and epithelial airway cells, and its expression is increased in the bronchial epithelium of asthmatic patients (Isacke and Yarwood 2002; Kumar et al. 2016; Lackie et al. 1997). However, CD44 expression is decreased in the airway smooth muscle cells of asthma and chronic obstructive pulmonary disease (COPD) patients, as shown by RT-PCR and Western blot analyses (Klagas et al. 2009). The de-polymerization of CD44 in the airway epithelial cells during inflammation also contributes to the hyper-secretion of mucus in asthma (Casalino-Matsuda et al. 2009). CD44 is also up-regulated in bronchial epithelial cells upon cellular damage in the airway, and the blockade of CD44 by neutralizing antibodies prevents cell migration (Leir et al. 2003). CD44 also interacts with other ERM (ezrin/radixin/moesin)-proteins (Yonemura et al. 1998). The GST-CD44 cytoplasmic domain binds to ERM-proteins with a high affinity, particularly to moesin, which has a  $K_D$  of  $9.3 \pm 1.6 \times 10^{-9}$  M (a smaller equilibrium dissociation constant ( $K_D$ ) indicates a higher affinity) (Hirao et al. 1996). Radixin binds to CD44 cytoplasmic peptides (292–363 residues) via the FERM domain, as demonstrated by structural studies (Mori et al. 2008). Because CD44 plays an important role in allergies, its interaction with ezrin has clinical implications as a potential pharmacological target (Katoh et al. 2011).

Similar to CD44, CD43 is a trans-membrane activation marker that interacts with ERM proteins and can contribute to asthma. CD43 regulates critical cellular functions, including T cell trafficking (Cannon et al. 2011). The activation of the T cell receptor (TCR) enhances the binding of ezrin to CD43, which induces the formation of a scaffold between the membrane and the cytoskeleton at the contact zone between the T cells and the antigen-presenting cells (APC) (Roumier et al. 2001). However, ezrin also colocalizes with CD43 in the opposite region, which is distal to the TCR engagement, suggesting that ezrin may contribute to the removal of inhibitory proteins from the immunological synapse during T cell activation (Allenspach et al. 2001).

### 3.3 VCAM

VCAM is expressed in tracheal smooth muscle and lung epithelial cells and modulates airway inflammation in asthma (Lin et al. 2015; da Silva et al. 2015). Immunoprecipitation assays have shown that VCAM-1 directly interacts with ezrin in the endothelial actin-rich docking structure, which mediates the leukocyte adhesion to the endothelium during inflammation in asthma (Barreiro et al. 2002). VCAM-1 signaling can be mediated by the advanced glycation end products (AGEs) receptor in pulmonary endothelial cells (Timothy et al. 2016). The N-terminal domain of ezrin binds to immobilized AGEs with a  $K_D$  value of  $5.3 \pm 2.1 \times 10^{-7}$  M. These results suggest that this interaction is specific and likely mediated by the exposed ezrin N-terminal domain because neither the full-length nor the C-terminal domain binds to AGEs. The binding to AGEs inhibits ezrin phosphorylation and the subsequent formation of tubules in kidney LLC-PK1 cells (McRobert et al. 2003).

In addition to VCAM, ezrin interacts with other critical adhesion molecules in airway smooth muscle cells, such as intercellular cell adhesion molecule (ICAM) (Arij et al. 2015). Ezrin interacts with both ICAM-1 and ICAM-2 (but not with ICAM-3), and the ezrin–ICAM-2 interaction has a  $K_D$  of  $3.3 \times 10^{-7}$  M (Heiska et al. 1998).  $\text{PIP}_2$  induces the interaction between ezrin and ICAM-1 and ICAM-2 (Heiska et al. 1998). ICAM-2 induces the phosphorylation of ezrin after the activation of Akt, which inhibits apoptosis in naive  $\text{CD}^{4+}$  cells (Perez et al. 2002). The crystal structures show that the ICAM-2 cytoplasmic domain binds to the groove of the phosphotyrosine binding (PTB)-like F3 subdomain of the N-terminal domain of radixin (Hamada et al. 2003).

### 3.4 DCC

The DCC is a part of the receptor complex of netrin-1 in the nervous system (Manhire-Heath et al. 2013). Netrin-1 regulates bleomycin-induced pulmonary



fibrosis and fibrocyte accumulation in the lungs. These results suggest that the DCC is implicated in respiratory diseases, such as asthma (Sun et al. 2016). The interaction between the ezrin N-terminal domain and the DCC cytoplasmic domain was originally shown in pull-down assays and later confirmed by co-immunoprecipitation in living DCC-transfected COS-1 cells (Martin et al. 2006). Netrin-1 induces the association between the DCC and ezrin and its subsequent phosphorylation (Antoine-Bertrand et al. 2011). Co-immunoprecipitation assays have shown that a DCC antibody pulls down ezrin after the stimulation of netrin-1 in IMR-32 cells (Antoine-Bertrand et al. 2011). Similar studies indicated that the transfection of DCC in *murine* neuroblastoma NG108–15 cells induces the binding of ezrin to PKA (Deming et al. 2015). This interaction has major cellular implications because the inhibition of ezrin protein expression abrogates the DCC-induced PKA activation and cellular growth (Deming et al. 2015).

### 3.5 *ERM-Binding Phosphoprotein 50 (EBP50)*

EBP50 (also known as the  $\text{Na}^+/\text{H}^+$  exchanger regulatory factor 1 (NHERF1)) is not an integral membrane protein, although it is recruited to the membrane and regulates membrane protein complexes. The binding of ERMs to certain membrane protein complexes is mediated by EBP50 (Fouassier and Fiorotto 2015). EBP50 is mainly expressed in airway epithelial cells and regulates vascular smooth muscle cell migration and cytokinesis in asthma (Fouassier et al. 2009; Baeyens et al. 2011). Ezrin binds EBP50 to complex with PAG (phosphoprotein associated with glycosphingolipid-enriched membrane microdomains) (Stokka et al. 2010). Ezrin appears to be an essential link that colocalizes PKA and *Src* with EBP50, which inhibits immune responses upon the activation of T cell (Cornez and Taskén 2010). EBP50 was first identified as an ezrin binding protein using affinity chromatography, and the C-terminal domain of EBP50 was sufficient for this association (Reczek and Bretscher 1998). A BIAcore analysis further confirmed the high affinity between ezrin and EBP50 with a  $K_D$  value of  $58.0 \pm 7.0 \times 10^{-9}$  M (Stokka et al. 2010). This interaction is hypothesized to be regulated by ezrin phosphorylation because EBP50 did not bind to full-length ezrin due to its intramolecular association blocking the N- and C-terminal domains (Reczek and Bretscher 1998).

## 4 Ezrin Modulates Signal Transduction Pathways

Ezrin modulates signal pathways mainly by connecting membrane receptor complexes to signaling pathways (Youn et al. 2009; Pore et al. 2015; Clucas and Valderrama 2014).

#### **4.1 *RhoA and the Rho-Associated Protein Kinase (ROCK) Pathway***

The RhoA/ROCK pathway is a critical signaling pathway that regulates both ezrin and airway smooth muscle contractions in asthma (Kosako et al. 2000). RhoA interacts with various effectors to regulate the downstream signaling pathways and the actin cytoskeleton (Schmieder et al. 2004; Anastasiadis et al. 2000). RhoA activates ezrin, which promotes the assembly of stress fibers and the polymerization of cortical actin in multiple cells, including fibroblasts, A431 and NIH3T3 cells (Mackay et al. 1997; Yonemura et al. 2002; Song et al. 2000). The activation of RhoA increases the intracellular levels of PIP<sub>2</sub>, which induces the phosphorylation of ezrin threonine-567 and therefore releases the ezrin C-terminal domain, allowing it to interact with F-actin (Castellani et al. 2012). The EGF-induced ezrin activation is mediated by RhoA because the inhibition of RhoA by fasudil (a potent Rho-kinase chemical inhibitor and vasodilator) prevents the activation of ezrin (Ma et al. 2013). Pretreatment with fasudil before the EGF stimulation decreased the phosphorylation of RhoA and the expression of ezrin in MDA-MB-231 cells. These results show that RhoA acts as an upstream factor that regulates ezrin (Ma et al. 2009). The inhibition of RhoA by C3 transferase (an exoenzyme that inhibits the addition of ADP-ribose moieties to Rho-like proteins) in cortical neurons abrogates the potential of netrin-1 to induce ERM phosphorylation via the DCC receptor (Lawrence et al. 2016).

Ezrin also interacts with Rho-GDI and enhances Rho activation (Takahashi et al. 1997; Nethe and Hordijk 2010). The binding of ezrin to Rho-GDI contributes to the activation of RhoA in MDCK cells after stimulation with podocalyxin (Schmieder et al. 2004). Once activated, RhoA maintains the ezrin activation and allows the connection of podocalyxin to actin at the apical cell membrane (Schmieder et al. 2004). Clinical evidence supports the relationship between RhoA and ezrin because more than 60% of ezrin-positive osteosarcomas show RhoA overexpression (Chiappetta et al. 2014). RhoA colocalizes with ezrin at the membrane ruffles of *human* endothelial cells that contribute to cellular migration during development (Menager et al. 1999). The phosphorylation of podocalyxin prevents its binding to ezrin and its subsequent dissociation from Rho-GDI. Thus, RhoA prevents the phosphorylation of ezrin by blocking its C-terminal actin-binding domain and inducing the dissociation of the podocalyxin/ezrin complexes from the actin cytoskeleton (Fukasawa et al. 2011). Treatment with podoplanin activates RhoA, which induces an epithelial mesenchymal transition in MDCK cells, and the activation of RhoA during the epithelial mesenchymal transition requires the binding of ezrin to the cytoplasmic tail of podoplanin (RKMSGRYSP) (Martin-Villar et al. 2006). The blockade of ezrin via RNA interference reduced the migration of ectopic endometrial cells and decreased the expression levels of RhoA and ROCK. These results suggest that the Ezrin/RhoA/ROCK pathway is a potential therapeutic target for the treatment of endometriosis (Jiang et al. 2012).

ROCK regulates the cytoskeleton by phosphorylating the myosin regulatory light chain at serine-19 during cytokinesis (Totsukawa et al. 2000). Recent studies have indicated that ROCK regulates the phosphorylation of ezrin, but some of these results are controversial. The ROCK inhibitor Y27632 prevents the phosphorylation of ezrin threonine-567 and its binding to the cytoskeleton after the transfection of RhoGEFs Net and Dbl in NIH3T3 fibroblasts (Tran Quang et al. 2000). However, other studies have indicated that the ROCK inhibitors Y27632 and HA1077 enhanced the total phosphorylation of ezrin in *human* glioblastoma U251 cells during cytokinesis (Kosako et al. 2000). These results are consistent with previous study showing that cisplatin induces the phosphorylation of ezrin by ROCK (Rebillard et al. 2010).

## 4.2 Protein Kinase A (PKA) Pathway

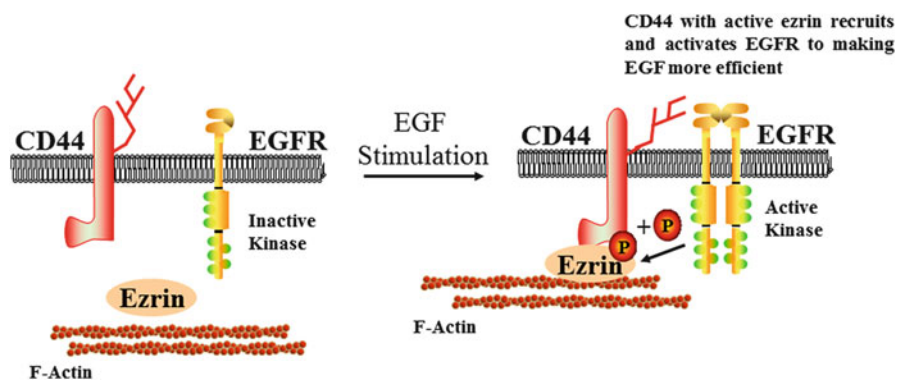
The cAMP/PKA pathway regulates ezrin phosphorylation, airway smooth muscle cell contraction, and cytokine production in asthma (Horvat et al. 2012). Ezrin is highly expressed in *human* airway smooth muscle cells and modulates  $\beta_2$ -adrenergic receptor signaling and muscle contraction in asthma (Horvat et al. 2012). Ezrin promotes the interaction between PKA and other proteins, such as the chloride channel cystic fibrosis trans-membrane regulator (CFTR), the transporter  $\text{Na}^+/\text{H}^+$  exchanger type 3 (NHE3), and the scaffold protein EBP50 (Vallée 2000; Soares et al. 2016). Ezrin is associated with cystic fibrosis, and several investigators have reported its binding to F508del CFTR, which is the most common mutation associated with this disease. In primary cystic fibrosis airway cells, the phosphorylation of ezrin threonine-567 enhances its binding to F508del CFTR and the actin cytoskeleton, activates the cAMP/PKA pathway, and rescues the F508del CFTR-dependent chloride secretion (Abbattiscianni et al. 2016). In *human* airway epithelial cells, the formation of the CFTR-ezrin complex increases the cAMP-mediated activation of CFTR (Ribas et al. 2007). In *human* airway smooth muscle cells, a disruption of the ezrin-PKA complex does not affect the  $\beta$ -agonist-induced accumulation of cAMP, but it increases the duration of plasma membrane-delineated cAMP (Horvat et al. 2012). Similarly, ezrin enhances PKA signaling during the  $\text{Na}^+/\text{H}^+$  exchanger activation. In the mammalian kidney, EBP50 enhances the phosphorylation of NHE3 by tethering NHE3 with the PKA-ezrin complex (Weinman et al. 2000). EBP50 also forms a complex with ezrin and the type-2 Na-Pi co-transporter in opossum kidney cells. Ezrin anchors PKA and triggers the phosphorylation of EBP50, which releases Npt2a and thereby inhibits the transport of phosphate (Wang et al. 2012). PKA promotes the phosphorylation of ezrin threonine-567 and its localization at the actin membrane ruffles, which activates the cAMP-induced exchange protein in HEK293T-EPAC1 cells (Parnell et al. 2015). Ezrin, CD99, soluble adenylyl cyclase, and PKA form a signaling complex at the endothelial junctions that regulates the movement of the recycling compartments to the site of the transendothelial migration (Watson et al. 2015). The

PKA-ezrin-Cx43 complex regulates the cAMP-induced gap junction connections (Pidoux and Tasken 2015). Ezrin binds to the adenosine A2b receptor and stabilizes the receptor complex after an adenosine stimulation in intestinal epithelial cells (Sitaraman et al. 2002). In contrast, ezrin, PKA, CFTR, and E3KARP (NHE3 kinase A regulatory protein) form a regulatory complex at the apical membranes of *human* airway epithelial cells to enhance the cAMP-induced activation of CFTR (Sun et al. 2000). Because the cAMP/PKA pathway contributes to asthma, these results suggest that the modulation of this pathway by ezrin can contribute to asthma.

## 5 Clinical Implications of Ezrin in Asthma

Ezrin is considered a key regulator of airway cells by modulating membrane–cortex interactions. The clinical implications of ezrin in asthma are growing and recent results suggest that ezrin is a potential therapeutic target in asthma. First, ezrin may participate in the repair of the bronchial epithelium during the early stages of asthma (Fig. 3). EGF is a key factor in bronchial epithelial repair and CD44 enhances the repair efficiency (Holgate 2000). The epithelial 3v isoform of CD44 is overexpressed in damaged tracheal epitheliums. Thus, EGF can induce the phosphorylation of ezrin, which links CD44 to the cortical actin cytoskeleton (Holgate 2000). Ezrin has also been reported to induce the secretion of mucin 5 AC (a typical feature of airway remodeling in asthma) after a neutrophil elastase attack in *human* airway epithelial cells.

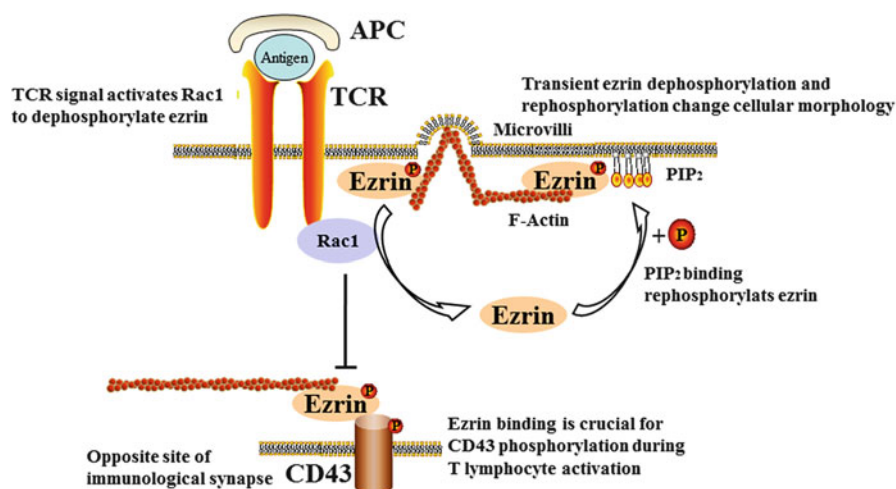
Second, ezrin appears to be required for the activation, morphological change, and apoptosis of T lymphocytes in asthma (Burkhardt et al. 2008). Ezrin is recruited



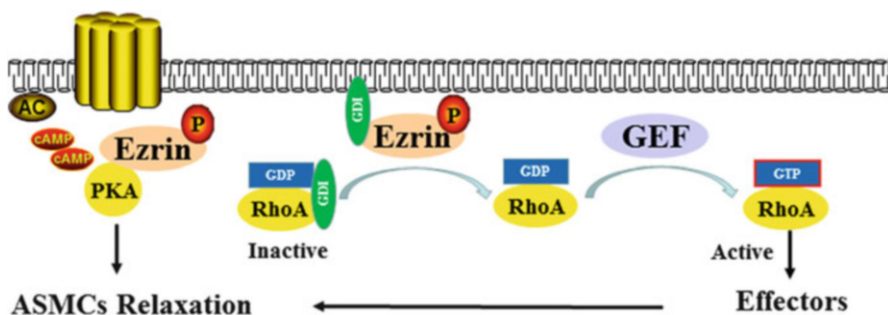
**Fig. 3** Proposed model of the participation of ezrin in the repair of the bronchial epithelium in asthma. EGF stimulation phosphorylates ezrin, which links CD44 to the cortical actin cytoskeleton. The interaction between CD44 and the EGFR enhances the repair efficiency of the airway epithelium. *P* phosphorylation, *EGF* epidermal growth factor, *EGFR* epidermal growth factor receptor

to the immunological synapse between the T lymphocytes and APC (Roumier et al. 2001). TCR activates Rac1 to dephosphorylate ezrin, and the phosphorylation of ezrin can be restored by  $PIP_2$  binding (Burkhardt et al. 2008). This regulation of ezrin collapses the microvilli, detaches the membrane from the actin cytoskeleton, and modulates the morphology of T lymphocytes (Fig. 4, upper panel) (Brown et al. 2003). Ezrin also plays an important role in the distal pole complex (DPC) at the opposite site of the immunological synapse (Allenspach et al. 2001; Shaffer et al. 2009). Ezrin binding is crucial for the phosphorylation of CD43 during T lymphocyte activation (Cannon et al. 2011). Similarly, the activation of Rac1 induces ezrin dephosphorylation and regulates its binding to membrane receptors at the DPC of T lymphocytes (Fig. 4, lower panel) (Burkhardt et al. 2008; Nijhara et al. 2004). Chemokine stimulation induces the dephosphorylation of ezrin, which triggers a rapid microvillar collapse in T lymphocytes, leading to the arrest of circulating T lymphocytes (Brown et al. 2003). Meanwhile, ezrin antisense oligonucleotides protect T lymphocytes from CD95-mediated apoptosis (Parlato et al. 2000). These results suggest that ezrin plays a role in T lymphocyte apoptosis and has clinical implications in asthma.

Third, ezrin can control the contraction of airway smooth muscle cells. Phosphorylated ezrin colocalizes with internalized  $\beta_2$ -adrenergic receptors and increases the re-sensitization of these receptors to cAMP stimulation, thus relaxing the airway smooth muscle cells (Horvat et al. 2012; Cant and Pitcher 2005). Furthermore, phosphorylated ezrin binds to Rho-GDI, enhances RhoA activation, and induces



**Fig. 4** Involvement of ezrin in the activation of T lymphocytes in asthma. At the immunological synapse, a TCR signal activates Rac1, which dephosphorylates ezrin, while binding to  $PIP_2$  re-phosphorylates ezrin. This transient dephosphorylation and re-phosphorylation of ezrin can change the cellular morphology of T lymphocytes. Ezrin interacts with CD43 during T lymphocyte activation at the DPC. *P* phosphorylation, *TCR* T cell receptor, *PIP<sub>2</sub>* phosphatidylinositol 4,5-bisphosphate, *APC* antigen-presenting cells, *Rac1* ras-related C3 botulinum toxin substrate 1, *DPC* distal pole complex



**Fig. 5** Possible mechanism of the ezrin-mediated regulation of the contraction and relaxation of airway smooth muscle cells. The PKA-Ezrin complex is the effector of  $\beta_2$ -adrenergic receptor signaling in the regulation of the contraction and relaxation of ASMCs. Phosphorylated ezrin binds to Rho-GDI, which enhances the activation of RhoA and induces the relaxation of ASMCs. *P* phosphorylation, *AC* adenylate cyclase, *cAMP* cyclic adenosine monophosphate, *PKA* protein kinase A, *GDP* guanosine diphosphate, *GDI* GDP dissociation inhibitor, *GEF* guanine nucleotide exchange factor, *GTP* guanosine-5'-triphosphate

airway smooth muscle cells relaxation (Fig. 5) (Takahashi et al. 1997; Fukata et al. 2001).

Altogether, these results suggest that ezrin can contribute to asthma by affecting bronchial epithelium repair, T lymphocyte regulation, and airway smooth muscle cell relaxation.

## 6 Conclusion and Perspectives

Ezrin is a critical protein that orchestrates membrane receptor complexes and intracellular transduction pathways. Ezrin is also a complex protein in terms of its structural features and functional regulation. Ezrin has multiple phosphorylation sites, but the biological and clinical implications of some of these sites remain unknown. Ezrin interacts with key receptors and adaptors, which modulates their signaling pathways, including the RhoA/ROCK and the cAMP/PKA pathways, in critical cellular processes associated with asthma. The clinical implications of ezrin in asthma are growing, and recent results support that ezrin is a potential therapeutic target for the treatment of asthma. These results warrant future studies to elucidate the interactions between ezrin and membrane receptor complexes and the regulation of airway cells in physiological and clinical conditions, such as asthma.

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## Competing Interests

The authors declare that they have no conflicts of interest.

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# Iron-Sulfur Protein Assembly in Human Cells



**Prasenjit Prasad Saha, Vinaya Vishwanathan, Kondalarao Bankapalli, and Patrick D'Silva**

**Abstract** Iron-sulfur (Fe-S) clusters serve as a fundamental inorganic constituent of living cells ranging from bacteria to human. The importance of Fe-S clusters is underscored by their requirement as a co-factor for the functioning of different enzymes and proteins. The biogenesis of Fe-S cluster is a highly coordinated process which requires specialized cellular machinery. Presently, understanding of Fe-S cluster biogenesis in human draws meticulous attention since defects in the biogenesis process result in development of multiple diseases with unresolved solutions. Mitochondrion is the major cellular compartment of Fe-S cluster biogenesis, although cytosolic biogenesis machinery has been reported in eukaryotes, including in human. The core biogenesis pathway comprises two steps. The process initiates with the assembly of Fe-S cluster on a platform scaffold protein in the presence of iron and sulfur donor proteins. Subsequent process is the transfer and maturation of the cluster to a bonafide target protein. Human Fe-S cluster biogenesis machinery comprises the mitochondrial iron-sulfur cluster (ISC) assembly and export system along with the cytosolic Fe-S cluster assembly (CIA) machinery. Impairment in the Fe-S cluster machinery components results in cellular dysfunction leading to various mitochondrial pathophysiological consequences. The current review highlights recent developments and understanding in the domain of Fe-S cluster assembly biology in higher eukaryotes, particularly in human cells.

**Keywords** Chaperones • Fe-S biogenesis • Iron-sulfur clusters • Iron-transfer • Mitochondria

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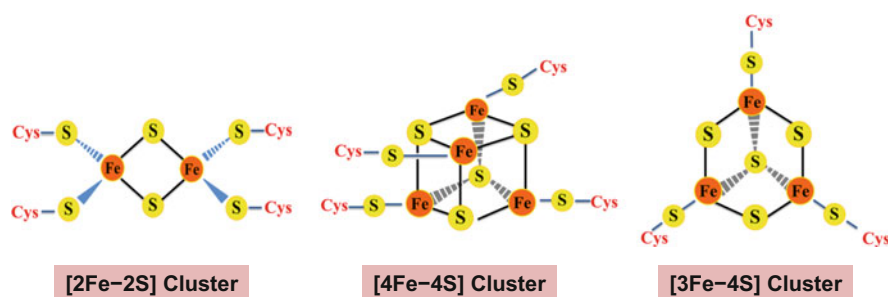
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## 1 Introduction: Iron Sulfur (Fe-S) Clusters Are Multifunctional Co-factor of Proteins

Biological activity of a protein is often performed by the assistance of a non-protein chemical component called co-factor (Wittung-Stafshede 2002). A plethora of different co-factors for proteins have been identified till date that enable a target protein to function efficiently. Among these, iron-sulfur (Fe-S) clusters are the oldest and ubiquitous co-factors known, that are present in all forms of life (Beinert 2000). Until date, more than 200 different types of enzymes or proteins are reported to harbor Fe-S clusters (Bandyopadhyay et al. 2008a). These clusters orchestrate a wide range of cellular functions in different environmental conditions. The critical role of Fe-S cluster is to perform electron transfer reactions in different biological processes, particularly during oxidative phosphorylation (OXPHOS) (Hatefi 1985). In addition to their core function, Fe-S clusters also act as catalytic centers, regulators of gene expression, and sensors of iron or oxygen (Beinert et al. 1997). These clusters are integrated into proteins through coordination of the iron atoms by cysteine or histidine residues, although in complex Fe-S clusters, alternative ligands are utilized (Meyer 2008). Despite the chemical simplicity of Fe-S clusters, their biogenesis in living cells is a highly intricate and coordinated process. Although several steps of the core biogenesis process are well established through various studies, many facts still remain unascertained. Since mammalian cells harbor several essential proteins containing Fe-S clusters for distinct biological functions, understanding their biogenesis draws significance in modern biology.

## 2 Structural Overview of Fe-S Clusters

Fe-S clusters are simplistically composed of iron atom either in ferrous ( $\text{Fe}^{2+}$ ) or ferric ( $\text{Fe}^{3+}$ ) form and inorganic sulfide ( $\text{S}^{2-}$ ) ions (Rees and Howard 2003). However, in rare cases, presence of additional metals or co-factors is reported. Based on the number of iron and sulfur ions, and their three-dimensional geometry, Fe-S clusters are mainly classified into two types. The simplest form of Fe-S cluster is the rhombic  $[\text{2Fe-2S}]$  cluster. It constitutes two iron atoms bridged by two sulfide ions resulting in one inorganic complex with unique spectroscopic properties (Fig. 1). In most occurrences, the iron atoms of  $[\text{2Fe-2S}]$  clusters are coordinated to proteins by four cysteine residues (e.g., ferredoxins). However, in certain cases, they are anchored to proteins by two cysteine and two histidine residues (e.g., Rieske Fe-S protein). The  $[\text{2Fe-2S}]$  clusters can exist in two oxidation states;  $[\text{2Fe-2S}]^{2+}$  and  $[\text{2Fe-2S}]^+$ , and mostly function in one-electron-transfer reactions. The second major type of Fe-S clusters is the cubane form of  $[\text{4Fe-4S}]$  clusters, which consist of four iron and sulfide ions placed at the vertices of a cubane-type structure with iron and sulfur occupying at the alternating corners of the cube (Fig. 1). Due to the shorter distance between the iron atoms, the structure is usually distorted. The  $[\text{4Fe-4S}]$  clusters can exist in four oxidation states;  $[\text{4Fe-4S}]^{3+}$ ,  $[\text{4Fe-4S}]^{2+}$ ,  $[\text{4Fe-4S}]^+$ , and  $[\text{4Fe-4S}]^0$ . These clusters are anchored to protein, mainly through cysteine residues and are present in proteins such as aconitase, ferredoxins, sulfite reductase, DNA glycosylase, etc. Some proteins such as subunits of complexes I and II of the respiratory chain contain  $[\text{3Fe-4S}]$  clusters in which three sulfide ions bridge two iron ions each, while the fourth sulfide bridges three iron ions. The  $[\text{3Fe-4S}]$  clusters can exist in four oxidation states:  $[\text{4Fe-4S}]^+$ ,



**Fig. 1** Types of Fe-S clusters. There are generally three types of Fe-S clusters. The simplest one is  $[\text{2Fe-2S}]$  cluster (*rhombic*), which is constituted of two iron ions (*red*) bridged by two sulfide (*yellow*) ions. Second one is  $[\text{4Fe-4S}]$  cluster (*cubane*) which consists of a *cubic* structure with iron and sulfur occupying alternating corners of the *cube*. The *cube* is usually distorted due to the shorter distance between the iron ions. Some proteins harbor  $[\text{3Fe-4S}]$  clusters in which one corner of the *cube* which contains iron is unoccupied. Fe-S clusters are usually integrated into protein backbone through coordination of the iron atoms primarily via cysteine or histidine residues. However, in more complex Fe-S clusters alternative ligands like asparagine, arginine, serine and functional groups like CO and  $\text{CN}^-$  have been identified



$[3\text{Fe-4S}]^0$ ,  $[3\text{Fe-4S}]^-$ , and  $[3\text{Fe-4S}]^{2-}$  (Fig. 1) (Johnson et al. 2005; Orme-Johnson 1973). Moreover, presence of a double cubane  $[8\text{Fe-7S}]$  cluster is also identified. These clusters are found only in nitrogenases and have the potential to act as a two-electron carrier (Rees 2002). They are anchored to proteins via cysteine residues, however in certain cases coordination through amino acids like histidine, arginine, serine, asparagine and non-protein ligands such as homocitrate, CO and  $\text{CN}^-$  functional groups (Ribbe et al. 2014). In general, although certain specific amino-acid side chains are frequently utilized as ligand for the coordination process, however, precise consensus sequence for binding Fe-S cluster in protein is still enigmatic. Fe-S clusters also exhibit a unique ability for structural inter-conversion, undergo oxidative degradation, and participate in ligand-exchange reactions. Reactive Oxygen Species (ROS), including superoxide ( $\text{O}_2^-$ ) and nitric oxide (NO) are capable of oxidizing these clusters, which leads to inter-conversion or disassembly of the clusters. Although there are well-characterized examples of  $[4\text{Fe-4S}] \leftrightarrow [3\text{Fe-4S}]$  and  $[4\text{Fe-4S}] \leftrightarrow [2\text{Fe-2S}]$  inter-conversion, there is no well-known example of inter-conversion between all three structures (Rees 2002; Johnson et al. 2005; Rees and Howard 2003; Frazzon and Dean 2003; Merchant and Dreyfuss 1998).

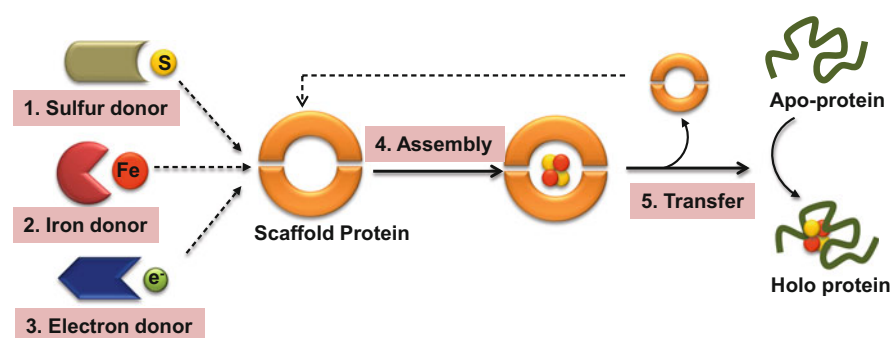
### 3 Diverse Biological Functions Assigned to Fe-S Clusters

The biological functions of Fe-S clusters can be grouped into three categories, namely electron transfer, enzyme catalysis, and regulation of biological processes (Beinert and Kiley 1999). The most common and critical function of Fe-S cluster is electron transfer in various redox reactions. This function relies on the property of the iron atom of the Fe-S clusters to switch between the reduced (ferrous  $\text{Fe}^{2+}$ ) and oxidized (ferric  $\text{Fe}^{3+}$ ) state. Thus, Fe-S clusters can accept electron from one species and transfer it to another, during electron transfer reactions (Brzoska et al. 2006). Due to their unique structural features, Fe-S clusters can attain redox potential from  $-500$  to  $+300$  mV within a particular proteinaceous surrounding, rendering them as excellent donor or acceptor of electrons (Meyer 2008). Several proteins participate in redox reactions by virtue of possessing different types of Fe-S clusters. For example, proteins involved in electron transfer reactions are complex I, complex II, complex III of the respiratory chain, ferredoxins of mitochondria and plant, nitrogenase of bacteria and photosystem I of plants (Rees and Howard 2000; Buckel et al. 2004; Dai et al. 2000). Furthermore, several Fe-S cluster containing proteins participate in enzyme catalysis reactions. The classical example of catalysis reaction by Fe-S cluster containing enzyme is aconitase, which harbors  $[4\text{Fe-4S}]$  cluster and catalyzes the conversion of citrate to isocitrate in the citric acid cycle. Non-protein coordinated iron at one edge of the  $[4\text{Fe-4S}]$  cluster of aconitase serves as a Lewis acid and assists in dehydration reaction leading to removal of water molecule from the substrate. Other examples of Fe-S clusters containing proteins involved in catalysis reactions are biotin synthase, lipoate synthase, ferrochelatase, etc. (Beinert and Kennedy 1993; Jarrett 2005; Dailey

2002). The third general function of Fe-S cluster is sensing extracellular or intracellular physiological changes thereby successively regulating specific gene expression. The chemical lability of Fe-S clusters and their pronounced sensitivity to oxidative damage make them proficient sensors for the changes in environmental or intracellular conditions at different stages of gene expression. In particular, regulation of gene expression in bacteria by the Fe-S cluster containing transcription factors, SoxR, IscR, and FNR occur at a transcriptional level (Kiley and Beinert 2003), whereas mammalian gene expression regulation by Fe-S cluster containing protein, iron regulatory protein 1 (IRP1) is performed at a post-transcriptional level. The switch between activation and repression of these Fe-S clusters containing transcription factors depends upon the physical presence or absence of Fe-S cluster (FNR and IscR) or redox state of the Fe-S cluster (SoxR) present in them. In case of IRP1 which harbors a [4Fe-4S] cluster, under iron-replete condition it functions as an aconitase enzyme. At the same time, under iron deprivation conditions, IRP1 in the absence of the Fe-S cluster regulates the expression of genes that are involved in iron-uptake, storage, and distribution (Kiley and Beinert 2003; Hentze et al. 2004; Pantopoulos 2004; Lill et al. 2006; Lill 2009; Beinert and Kiley 1999).

## 4 Biosynthetic Principle of Fe-S Cluster Biogenesis

Despite the complexity and intricacy of Fe-S clusters synthesis in living cells, the basic biosynthetic principle remains similar across species. The overall biogenesis process can be split into two major events; (1) the *de novo* assembly of Fe-S cluster onto a scaffold protein and (2) transfer and integration of the assembled Fe-S cluster from the scaffold protein to a recipient apo-protein (Fig. 2).



**Fig. 2** Different steps of Fe-S cluster biogenesis. The biogenesis of Fe-S cluster constitutes of five basic steps. Cysteine desulfurase protein acts as sulfur donor and transfers sulfur from cysteine to the scaffold protein (step 1). The transfer of iron requires an iron donor, as iron is unlikely to be free in solution (step 2). Dedicated electron transport machinery delivers electron for reduction of sulfur to sulfide (step 3). Subsequently, the raw components assemble as Fe-S clusters on a conserved scaffold protein (step 4). The assembled Fe-S clusters thereafter get targeted to an apo-protein (step 5)

The first major event involving the de novo assembly of Fe-S cluster requires sulfur as a raw material and a sulfur donor protein. A cysteine desulfurase enzyme performs the transfer process by releasing sulfur from a free cysteine amino-acid and converting it to alanine. As an intermediate, a persulfide is formed on a conserved cysteine residue of the enzyme. This sulfur in the form of persulfide intermediate successively gets transferred to the scaffold protein directly or with the aid of helper proteins (Lill and Muhlenhoff 2006; Chandramouli et al. 2007; Zheng et al. 1993; Kaiser et al. 2000; Fontecave and Ollagnier-de-Choudens 2008). The second essential raw material required for the Fe-S cluster biogenesis process is iron. The delivery of iron atom to the core process is a tightly controlled and regulated pathway involving a specific iron donor that promotes a close interaction with the components of the Fe-S cluster biogenesis system (Fig. 2) (Lill 2009; Hentze et al. 2010; Yoon and Cowan 2003). The third major component required for the building of Fe-S cluster is an electron donor. Sulfur in cysteine remains in  $S^0$  state whereas in Fe-S cluster it exists in the reduced form ( $S^{2-}$ ). Hence, the reduction of sulfur ( $S^0$ ) to sulfide ( $S^{2-}$ ) requires an electron donor. A dedicated electron donor system accomplishes electron transfer for the biogenesis process (Lange et al. 2000; Li et al. 2001; Muhlenhoff et al. 2002a). A dedicated scaffold protein serves as a platform where the Fe-S clusters are assembled with the assistance of additional helper proteins. The scaffold protein has conserved cysteine residues that help the protein to bind Fe-S cluster in a transient manner, such that the cluster can be transferred to a target apo-protein efficiently (Muhlenhoff et al. 2003a; Raulfs et al. 2008).

The second major event involves transfer of assembled Fe-S cluster from scaffold protein to an apo-protein. A dedicated set of specific protein factors are required for the transfer process. These transfer proteins induce dissociation of cluster from the scaffold protein, ensuring precise transfer of the cluster to a *bonafide* apo-protein and facilitate its accurate insertion at the appropriate acceptor sites of client proteins (Fig. 2) (Chandramouli and Johnson 2006; Bonomi et al. 2008).

## 5 Machinery for Fe-S Clusters Synthesis: Bacteria to Higher Eukaryotes

There are distinct machineries identified for the biogenesis of Fe-S clusters in living cells which follow a similar basic principle of cluster synthesis. In bacteria, three machineries are identified that are capable of assembling Fe-S proteins, viz. nitrogen-fixation (NIF) machinery, iron-sulfur cluster (ISC) assembly machinery, and sulfur-mobilization (SUF) machinery. The NIF machinery present in nitrogen-fixing bacteria (e.g., *Azotobacter vinelandii*) is specialized in synthesizing and assembling Fe-S clusters in the nitrogenase enzyme complex. On the other hand, ISC and SUF machinery are responsible for the synthesis of housekeeping Fe-S

proteins under normal and oxidative stress condition, respectively (Lill et al. 2006). It is generally assumed that during evolution, bacterial Fe-S cluster machineries were transferred to eukaryotes by endosymbiosis. In eukaryotes, Fe-S cluster biogenesis machinery is present in mitochondria, plastid (in plants) and cytosol (Balk and Lobreaux 2005). During evolution, proteins homologous to bacterial ISC assembly machinery and SUF machinery were retained in the mitochondria and plastid, respectively. *Saccharomyces cerevisiae* is a well-known model organism utilized to understand the mechanism of eukaryotic Fe-S cluster biogenesis. The Fe-S cluster biogenesis process in yeast occurs mainly in the mitochondrial compartment. However, cytosolic and nuclear Fe-S cluster biogenesis system have also been reported in different studies (Paul and Lill 2015). The assembly of Fe-S cluster in the eukaryotic cytosol and nucleolus requires the assistance of mitochondrial ISC assembly and export machinery. Maturation of these extra mitochondrial Fe-S protein is also dependent on the essential cytosolic Fe-S cluster assembly (CIA) machinery, which is present in virtually all eukaryotes (Lill et al. 2006; Lill 2009; Lill and Kispal 2000; Balk and Lobreaux 2005; Lill and Muhlenhoff 2006).

## 6 Biogenesis of Fe-S Clusters in Human

Although the biogenesis of Fe-S clusters is well studied in different model organisms, understanding human Fe-S cluster biogenesis has drawn attention in recent times, considering the close association of human diseases with impairment of Fe-S cluster biogenesis. Similar to other higher eukaryotes, mammalian cells possess both mitochondrial and extra mitochondrial machinery for Fe-S protein maturation, which follows similar basic steps of cluster synthesis.

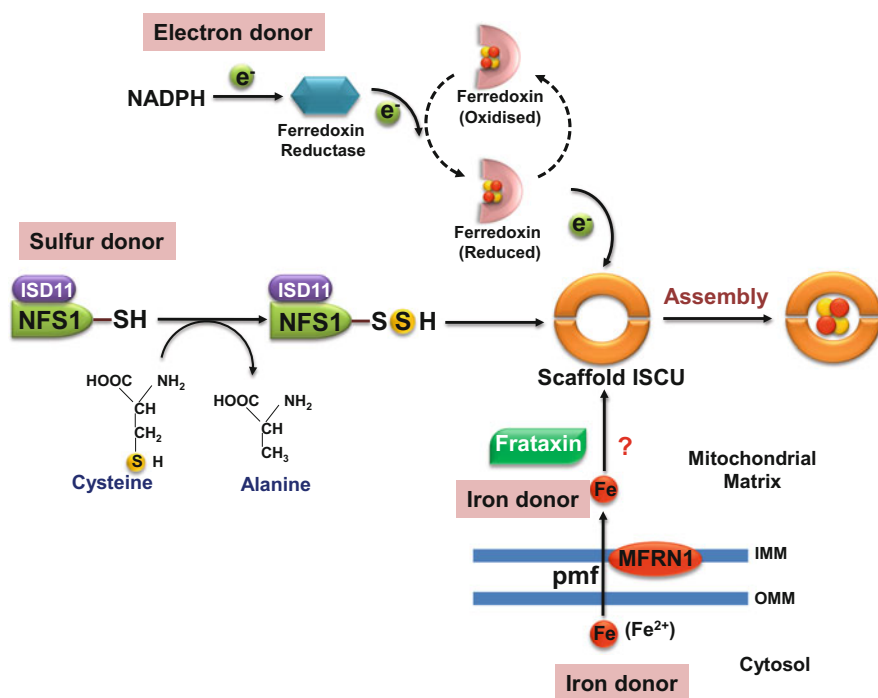
### 6.1 Mitochondrial Fe-S Cluster Biogenesis

In mammalian cells, mitochondria are the major organelles for the biogenesis of Fe-S clusters. Human mitochondrial matrix harbors well-conserved Fe-S cluster assembly (ISC) machinery, which synthesizes the major pool of overall cellular Fe-S cluster and assists extra mitochondrial cluster synthesis. The major steps of human mitochondrial Fe-S cluster biogenesis are described below.

#### 6.1.1 Sulfur Transfer

Sulfur transfer process is relatively well studied in the yeast model system. The function is performed by the cysteine desulfurase protein complex Nfs1-Isd11. Similarly, in human mitochondria, the process of sulfur transfer is assisted by cysteine desulfurase protein NFS1 (Nfs1 in yeast) with the assistance of ISD11

(Isd11 in yeast) protein. This pyridoxal phosphate (PLP) dependent NFS1 is a hydrophobic protein and localized to the mitochondrial matrix (Li et al. 1999; Kispal et al. 1999). Majority of human NFS1 protein is localized in the mitochondrial compartment, however smaller levels were also detected in the cytosolic and nuclear compartment (Biederbick et al. 2006). Human NFS1 obtains sulfur from free cysteine to generate a persulfide ( $-SSH$ ) intermediate at its active site cysteine residue (Cys 381) and in the process it converts free cysteine to alanine (Fig. 3) (Parent et al. 2015). The function of mitochondrial NFS1 is assisted by a low molecular weight mitochondrial matrix protein ISD11, which prevents it from self-aggregation (Shi et al. 2009). Besides, ISD11 also plays a major role in promoting persulfide intermediate formation at the active site cysteine of NFS1 (Pandey et al. 2012). The mechanism of sulfur transfer from NFS1 to the scaffold



**Fig. 3** Sulfur, iron, and electron transfer for assembly of Fe-S clusters. In human, cysteine desulfurase protein, NFS1 along with ISD11 serves a functional sulfur donor complex. NFS1 obtains sulfur from free cysteine and converts the amino acid to alanine. The sulfur remains as a persulfide ( $-SSH$ ) intermediate at the active site cysteine of NFS1. Furthermore, the sulfur is transferred to scaffold protein ISCU by an unknown mechanism. An electron transfer chain comprised of NADPH, ferredoxin reductase (FDR), and ferredoxin (FDX2) provides electron ( $e^-$ ) for reduction of sulfur ( $S^0$ ) to sulfide ( $S^{2-}$ ). Mitochondria imports ferrous iron ( $Fe^{2+}$ ) from cytosol in a membrane potential dependent manner which is facilitated by the inner membrane carrier proteins mitoferrin (MFRN1/2) and other unknown factors. The transfer of iron to scaffold protein is a less defined process. The iron-binding protein frataxin is believed to function as the putative iron donor

protein is not clearly understood. By considering the extreme toxicity associated with free sulfur, it is highly unlikely to be transferred to the scaffold protein in a free (soluble) form. Instead, sulfur probably transfers from the active site cysteine of NFS1 to a catalytically active acceptor cysteine residue of the scaffold protein directly or apparently via helper protein(s) (Agar et al. 2000; Smith et al. 2001; Urbina et al. 2001). The crystal structure of cysteine desulfurase protein from several bacterial species has been solved. The crystal structure of the dimeric protein showed that each monomer consists of two domains. One domain binds to the pyridoxal phosphate, and the comparatively smaller domain harbors the active site cysteine that transiently holds the sulfur as persulfide released from free cysteine (Kaiser et al. 2000; Cupp-Vickery et al. 2003). Although studies in yeast indicate that Isd11 is not necessary for the desulfurase activity of Nfs1 in vitro, Nfs1-Isd11 complex is the functional entry for transfer of sulfur to scaffold protein, in vivo (Wiedemann et al. 2006). Similar study on human ISD11 confirms that it plays a major role in NFS1-ISD11 sub-complex stability and in the absence of ISD11 protein, NFS1 undergoes aggregation and degradation (Saha et al. 2015). Moreover, it is reported that the N-terminal region of ISD11 plays an important role in interaction with NFS1 and helps in subcellular targeting of the protein (Friemel et al. 2017). Recently, it has been shown that ACP (Acyl Carrier Protein) interacts and stabilizes the NFS1-ISD11 complex (Van Vranken et al. 2016). Crystal structure of NFS1-ISD11-ACP complex reveals key residues that stabilize the interaction of ISD11 with NFS1 and ACP. Additionally, the quaternary structure adopted by the complex provides a rationale for the increased efficiency of sulfur transfer mediated by Frataxin (FXN) (Cory et al. 2017).

Depletion of mitochondrial NFS1 in human cell lines shows diminished cell viability and imposes abnormal changes in mitochondrial morphology. Moreover, the activities of mitochondrial as well as cytosolic Fe-S cluster containing enzymes are severely compromised upon NFS1 depletion, suggesting that NFS1 performs a critical role in Fe-S cluster biogenesis in human cells (Biederbick et al. 2006). On the other hand, depletion of ISD11 in human cell lines demonstrates severe Fe-S cluster biogenesis defects and deregulation of iron homeostasis (Shi et al. 2009).

Apart from sulfur donation for Fe-S cluster biogenesis process, NFS1 also plays a critical role in thio-modification of mitochondrial and cytosolic tRNAs. Additionally, cytosolic NFS1 assists in molybdenum cofactor biosynthesis by providing sulfur to MOCS3 (Marelja et al. 2008, 2013; Nakai et al. 2007; Wohlgamuth-Benedum et al. 2009).

### 6.1.2 Iron Transfer

The iron utilized for Fe-S cluster assembly process is transported from cytosol. The iron trafficking process from cytosol to scaffold-protein is a well-regulated phenomenon. Most of the studies to decipher the iron transfer steps are performed using a yeast model system which further aids in understanding the process in human cells. The entry of iron inside human mitochondria from cytosol is a mitochondrial

membrane potential dependent process, which is facilitated by carrier protein mitoferrin (MFRN1/2) (Mrs3 and Mrs4 in yeast) and further unknown factors (Lange et al. 1999; Muhlenhoff et al. 2003b; Zhang et al. 2005). Iron is transported into mitochondria in the reduced state ( $\text{Fe}^{2+}$ ). However, it is not clear in which form (free or chelated) iron enters into mitochondria. Equally, the state of iron which exists in the mitochondrial matrix before utilization for biogenesis of Fe-S cluster (or for heme synthesis) is also not well defined. At the same time, studies have demonstrated that human mitochondrial ferritin plays a crucial role in storing iron in a soluble, non-toxic, and readily available form. Ferritin takes up iron in the ferrous form ( $\text{Fe}^{2+}$ ) and deposits it as ferric hydroxides after oxidation. Nevertheless, release of iron from ferritin is not well established. Although mitochondrial ferritin plays a crucial role in cellular iron homeostasis, but it exhibits very restricted tissue-specific expression pattern. Mitochondrial ferritin levels are very high in testis, but very low levels are reported in iron-storage organs (Drysdale et al. 2002; Arosio and Levi 2010; Langlois d'Estaintot et al. 2004; Levi et al. 2001). The transfer of iron to the scaffold protein for the biogenesis of Fe-S cluster is probably facilitated by Frataxin (FXN), the human homologue of yeast Yfh1 (Fig. 3). Frataxin/yfh1 deficiency in the mitochondrial matrix is associated with compromised Fe-S protein activity, suggesting the importance of Frataxin/Yfh1 in the biogenesis process (Puccio et al. 2001; Chen et al. 2002; Muhlenhoff et al. 2002b). Purified Yfh1 protein is found to bind several atoms of iron with low affinity, although the specificity of binding is still debatable (Yoon and Cowan 2003; Aloria et al. 2004; Nair et al. 2004). Importantly, it has been reported that Yfh1 binds to scaffold protein Isu1 in an iron-dependent manner, implying that complex formation may facilitate iron transfer from yfh1 to Isu1 for the assembly of Fe-S cluster (Gerber et al. 2003). Recent reports highlight that Fe-S cluster assembly occurs through formation of multi-protein complexes of the scaffold protein and sulfur donor complex with the help of Frataxin. Study conducted in a mammalian system reveals that Frataxin binds to and stabilizes the ternary complex (ISCU/NFS1/ISD11) which in turn regulates the iron entry into the quaternary complex (ISCU/NFS1/ISD11/FXN). The enhancement in the desulfurase activity by FXN in the quaternary complex is achieved by inducing a conformational change, which positions NFS1 persulfide loop close to the cysteine residue on ISCU thereby increasing the cysteine turnover (Colin et al. 2012). Studies performed in vitro suggest that FXN functions as an allosteric activator by binding and stabilizing the C-terminal  $\alpha$ -helix of ISCU. This conformational change could be responsible for enhanced persulfide formation on NFS1 and transfer of sulfur from NFS1 to ISCU (Parent et al. 2015; Pandey et al. 2013; Bridwell-Rabb et al. 2014; Fox et al. 2015b).

### 6.1.3 Electron Transfer

The biogenesis of Fe-S cluster requires a steady supply of electrons ( $\text{e}^-$ ), which is provided by a dedicated electron transport chain present in the mitochondrial matrix, although the precise mechanism of electron transfer is not well understood

(Yan et al. 2015; Webert et al. 2014). The presence of the electron supplier is prerequisite for the reduction of sulfur ( $S^0$ ) formed during the cysteine desulfurase reaction to sulfide ( $S^{2-}$ ) which is present in Fe-S clusters. However, experimental verification for the sulfur reduction to sulfide is still to be established. Additionally, requirement of electron has also been suggested for the fusion of two [2Fe-2S] clusters to form a [4Fe-4S] cluster by reductive coupling (Chandramouli et al. 2007; Unciuleac et al. 2007). In humans, ferredoxin reductase FDXR (Arh1 in yeast) and ferredoxin FDX (Yah1 in yeast) form an electron transfer chain, which receive an electron from NADPH and successively transfer to scaffold protein for the assembly process (Fig. 3) (Lill et al. 2006). In yeast, depletion of Arh1 or Yah1 drastically affects the Fe-S cluster assembly process, suggesting their importance in the early events of Fe-S cluster biogenesis (Li et al. 2001; Muhlenhoff et al. 2003a; Lange et al. 2000). Similarly in human cell lines, knockdown of ferredoxin reductase FDXR results in reduced Fe-S cluster assembly and loss of cellular iron homeostasis (Shi et al. 2012). Human mitochondria possess two [2Fe-2S] cluster containing ferredoxins, FDX1 and FDX2. Reports suggest that FDX1 (also known as adrenodoxin) catalyzes reactions central to steroidogenesis, whereas FDX2 (also known as FDX1L) is involved in heme and Fe-S cluster biogenesis (Sheftel et al. 2010). Latest study in human cell lines reveals that FDX1 also participates in the formation of Fe-S cluster assembly on a scaffold protein similar to FDX2. Furthermore, depletion of FDX1 leads to diminished level of Fe-S proteins and loss of cellular iron homeostasis, suggesting the importance of FDX1 in Fe-S cluster biogenesis (Shi et al. 2012).

#### 6.1.4 Scaffold Protein for Fe-S Cluster Assembly Process

The central part of Fe-S protein biogenesis in mitochondria is de novo synthesis of the Fe-S clusters on a highly conserved scaffold protein. In human, mitochondrial matrix protein ISCU serves as a molecular scaffold for assembly of Fe-S clusters. However, in *Saccharomyces cerevisiae*, two orthologs of human ISCU, namely Isu1 and Isu2 are present in the mitochondrial matrix. Importantly, the *ISU1* and *ISU2* double deletion yeast mutants are inviable, thus signifying the importance of both Isu1/2 proteins in the Fe-S cluster biogenesis (Garland et al. 1999). The scaffold protein ISCU harbors three phylogenetically conserved cysteine residues that participate in the coordination process during the synthesis of Fe-S clusters. These cysteine residues are moderate to completely solvent accessible and are located adequately close to each other, such that they can bind to Fe-S clusters effectively (Ayala-Castro et al. 2008). The oligomeric state of the scaffold protein may play a significant contributing factor in terms of precise coordination geometry is concerned in order to anchor a Fe-S cluster. The first crystal structure of IscU from the hyperthermophilic bacterium, *Aquifex aeolicus* with a bound [2Fe-2S] cluster was reported to exist as a trimer (Shimomura et al. 2007, 2008). However, IscU from *Azotobacter vinelandii* and *Schizosaccharomyces pombe* exists as a dimer, retaining either one or two [2Fe-2S] centers or a single [4Fe-4S] cluster



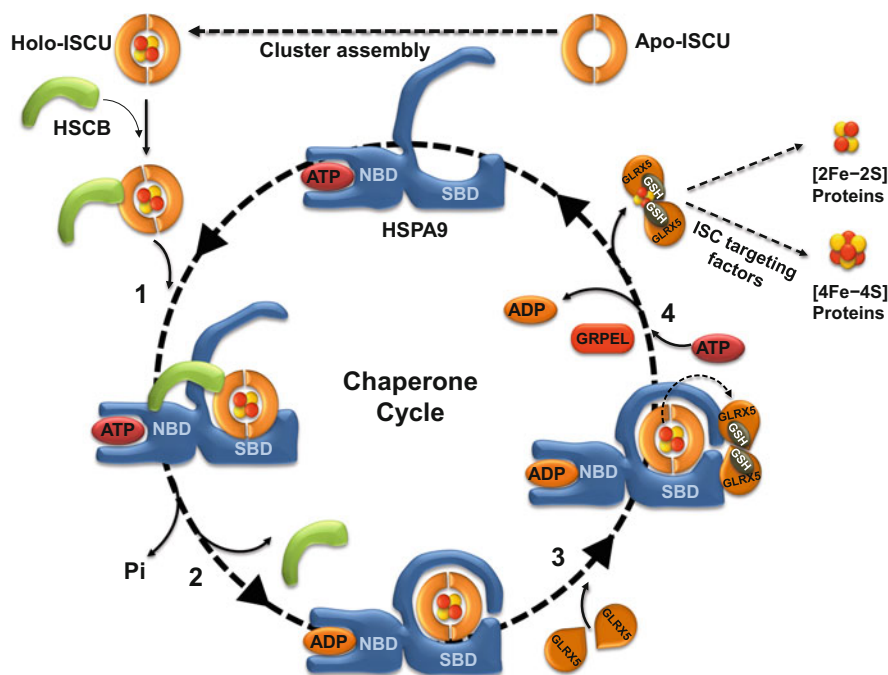
(Wu et al. 2002; Agar et al. 2000). Structurally, the significance of oligomeric state of the human ISCU or yeast Isu1 is not clearly defined but the monomeric form probably is not the functional entity to form Fe-S cluster. Recent biochemical evidences suggest that the apo-ISCU exists as a dimer, therefore, human ISCU probably participates as a dimer in the assembling of Fe-S clusters (Saha et al. 2014).

The chemistry pertinent to Fe-S cluster synthesis is poorly understood as it is challenging to decipher whether iron or sulfur binds first to the scaffold protein (Fontecave et al. 2005; Johnson et al. 2005). Further, detailed mechanism of the molecular catalytic events is not well characterized. Though, in vitro reconstitution experiments using yeast or bacterial protein partners for synthesizing Fe-S clusters on scaffold protein have provided preliminary evidences towards understanding the molecular mechanism, nonetheless, the overall molecular events associated with assembly process still remain largely elusive (Dutkiewicz et al. 2006; Prischi et al. 2010; Agar et al. 2000; Raulfs et al. 2008). Lately, in vitro reconstitution of Fe-S clusters using human protein partners on ISCU protein has also been accomplished. The human Fe-S cluster assembly complex (consists of NFS1, ISD11, Frataxin, and ISCU) catalyzes the synthesis of [2Fe-2S] clusters on ISCU protein in vitro, which further can be transferred to acceptor molecules (Fox et al. 2015a, b).

#### 6.1.5 Cluster Dissociation from Scaffold Protein

One of the critical events during the biogenesis process is the release of Fe-S cluster from scaffold protein, followed by targeting and integration into an apo-protein. This transfer process is mediated by a dedicated chaperone machinery comprising the mtHsp70, namely Mortalin or HSPA9 (Ssq1 in yeast) and the DnaJ-class of cochaperone, HSCB or HSC20 (Jac1 in yeast). In human mitochondria, the ADP/ATP nucleotide exchange factor, GRPEL (Mge1 in yeast) regulates the nucleotide-dependent binding of HSPA9 to client proteins (Naylor et al. 1998; Westermann et al. 1995). Studies performed in yeast model system showed that the J-protein Jac1 first binds to the Isu1 and subsequently targets to Ssq1. Yeast Isu1 with conserved LPPVK motif interacts with ATP-bound form of Ssq1 and the binding is further stabilized by the ATP-hydrolysis (Cupp-Vickery et al. 2004; Dutkiewicz et al. 2004). Although Ssq1 has a weak intrinsic ATPase activity, the ATP hydrolysis process is stimulated synergistically by Jac1 and Isu1 subcomplex, probably inducing a structural rearrangement in the Isu1 protein. This results in dissociation of Jac1 from the complex, thus further weakening the affinity of bound Fe-S cluster to Isu1, thereby enabling cluster dissociation (Dutkiewicz et al. 2003; Bonomi et al. 2011). The cycle of interaction is completed when ADP is exchanged for ATP by Mge1, and the components are released for utilization in the next cycle (Craig and Marszalek 2002; Lill et al. 2006, 2012; Lill 2009; Lill and Kispal 2000; Lill and Muhlenhoff 2005, 2008; Bandyopadhyay et al. 2008a; Rouault and Tong 2005).

Considering the high degree of sequence similarity of human mitochondrial chaperone machinery to the yeast orthologs, one could predict that the human Fe-S cluster biogenesis follows a similar chaperone cycle for targeting of Fe-S clusters to bona fide proteins (Fig. 4). Unlike yeast mitochondria which have three different mtHsp70 proteins (Ssc1, Ssq1 and Ecm10) dedicated for different functions, human mitochondria possess one mtHsp70, namely HSPA9, which performs plethora of functions, including protein import, folding, and Fe-S cluster biogenesis



**Fig. 4** Schematic model of dissociation of Fe-S cluster from ISCU, assisted by chaperones and GLRX5. The whole process of dissociation and transfer of the clusters from scaffold protein to a target consists of four basic steps. Holo-ISCU transiently bound to [2Fe-2S] cluster is targeted to ATP-bound Hsp70 chaperone HSPA9 with the help of Hsp40 co-chaperone HSCB (step 1). HSPA9 binds to conserved LPPVK motif of ISCU, thus stimulating the ATPase activity of HSPA9 in presence of HSCB. ATP hydrolysis results in an open to closed conformational state of the substrate binding domain (SBD) of HSPA9, resulting in a tight association of ADP-bound HSPA9 with ISCU and release of HSCB (step 2) (Kampinga and Craig 2010). The strong binding between HSPA9 and ISCU is believed to induce a conformational change in the scaffold protein, such that it weakens the binding affinity of the bound Fe-S cluster, thereby facilitating the cluster release. In the next step, monothiol glutaredoxin GLRX5 binds to HSPA9 near to ISCU. The association leads to cluster transfer from ISCU to GLRX5 which together with glutathione (GSH) binds to a [2Fe-2S] cluster (step 3). Nucleotide exchange factor, GRPEL1 exchanges ADP for ATP on HSPA9, thereby triggering conformational changes in its SBD from closed to opened state. This results in disassembly of HSPA9-ISCU complex and allows the proteins to start a new cycle (step 4). Holo-GLRX5 associated with [2Fe-2S] cluster transfers the cluster to [2Fe-2S] proteins and/or [4Fe-4S] cluster targeting factors (Uzarska et al. 2013)

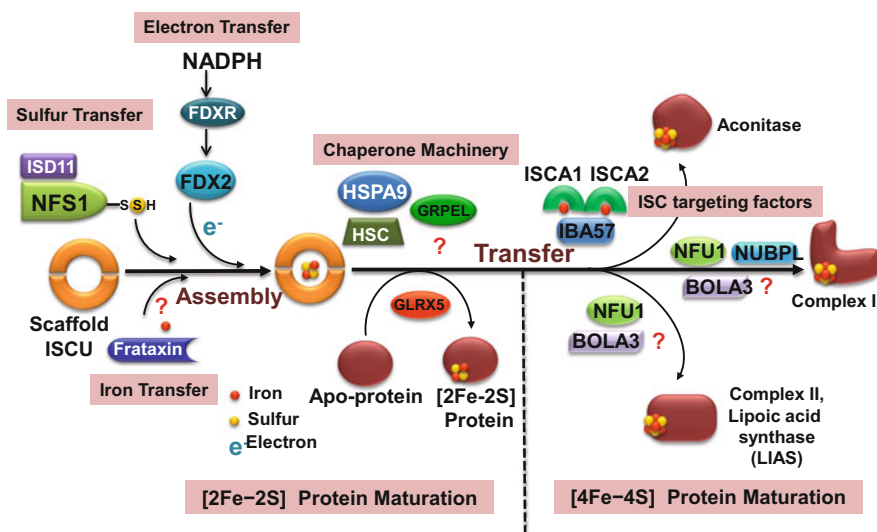
(Schilke et al. 2006; Pukszta et al. 2010; Amick et al. 2014; Goswami et al. 2010). Recent studies highlight that silencing HSPA9 in human cell lines results in diminished activity of Fe-S cluster containing enzymes, suggesting involvement of the protein in Fe-S cluster biogenesis (Maio et al. 2014). Multiple sequence alignment of human ISCU with different orthologs across different species revealed identical conserved LPPVK motif, which might play a similar role in mediating HSPA9 interaction during the assembly process (Cai et al. 2013).

Studies performed using human orthologous proteins demonstrated that HSC20 plays a critical role in Fe-S cluster biogenesis and interacts with the predicted partner proteins HSPA9 and ISCU (Schilke et al. 2006; Amick et al. 2014). Importantly, HSC20 along with ISCU stimulates the ATPase activity of HSPA9 (Amick et al. 2014), suggesting that human mitochondrial molecular chaperone system functions similar to the yeast counterpart. HSC20 is an unusual DnaJ type III protein which has an N-terminal tetra-cysteine metal binding domain (Uhrigshardt et al. 2010; Bitto et al. 2008). However, the role of this N-terminal metal binding domain in Fe-S cluster biogenesis is not well understood. Recently, additional novel function has been assigned for HSC20 in the process of transfer of clusters to specific target proteins. HSC20 possesses a unique ability to bind to conserved LYR-motifs present in Fe-S cluster recipient proteins, thus enhancing the specificity of Fe-S cluster delivery to the target apo-proteins (Maio et al. 2014).

### 6.1.6 Transfer of [2Fe-2S] Clusters

Transfer of Fe-S cluster after dissociation from scaffold protein to an apo-protein or to any cluster targeting factors is a crucial step during Fe-S cluster biogenesis. The monothiol glutaredoxin GLRX5, a human homologue of yeast Grx5, plays an important role in the transfer of [2Fe-2S] cluster to target proteins. Although early experiments in yeast and bacteria demonstrated that Grx5 involves in facilitating the transfer of [2Fe-2S] clusters from the scaffold to target proteins, the exact molecular details are still unclear (Shakamuri et al. 2012). According to the findings, Grx5 forms a dimer which coordinates a [2Fe-2S] cluster via the active site cysteine residue with the assistance of two non-covalently bound glutathione (GSH) (Bandyopadhyay et al. 2008b; Johansson et al. 2011; Picciocchi et al. 2007). Moreover, Grx5 binds to Ssq1 adjacent to the binding site of Isu1. This allows transfer of Isu1 bound Fe-S cluster to Grx5 efficiently, which further mediates transfer of cluster to target proteins (Uzarska et al. 2013). Through this transient Fe-S cluster carrier, Grx5 is capable of binding both ATP- and ADP-bound forms of Ssq1, but interacts predominantly in the ADP-bound form of Ssq1. Importantly, deletion of Grx5 exhibits a viable growth phenotype in yeast, suggesting that Grx5-mediated cluster transfer is not essential, *in vivo*. Therefore, the [2Fe-2S] clusters could be transferred directly or indirectly with the help of other unknown mediators (s), from holo-Isu1 to the client proteins (Fig. 4) (Rodriguez-Manzanique et al. 1999, 2002). On the other hand, study on human GLRX5 demonstrated that deficiency of GLRX5 protein in human cells causes compromised Fe-S cluster

biogenesis and impairs cellular iron homeostasis, signifying the importance of GLRX5 in Fe-S cluster biogenesis (Ye and Rouault 2010). Crystal structure of human GLRX5 revealed a tetrameric organization bound to two [2Fe-2S] clusters and four GSH molecules. In contrast, new findings suggest that structure of holo-GLRX5 in solution solved by NMR spectroscopy represents a symmetric dimer bridged by a [2Fe-2S] cluster with one GSH molecule attached per subunit. This organization of holo-GLRX5 allows efficient cluster transfer due to improved accessibility of the cluster to client proteins and serves as a better model as compared to the tetrameric structural organization found in the crystal structure (Banci et al. 2014). Although studies suggest that GLRX5 plays a major role in transfer of [2Fe-2S] clusters, nonetheless, the process is complex in nature and remains to be resolved further in details (Fig. 5).

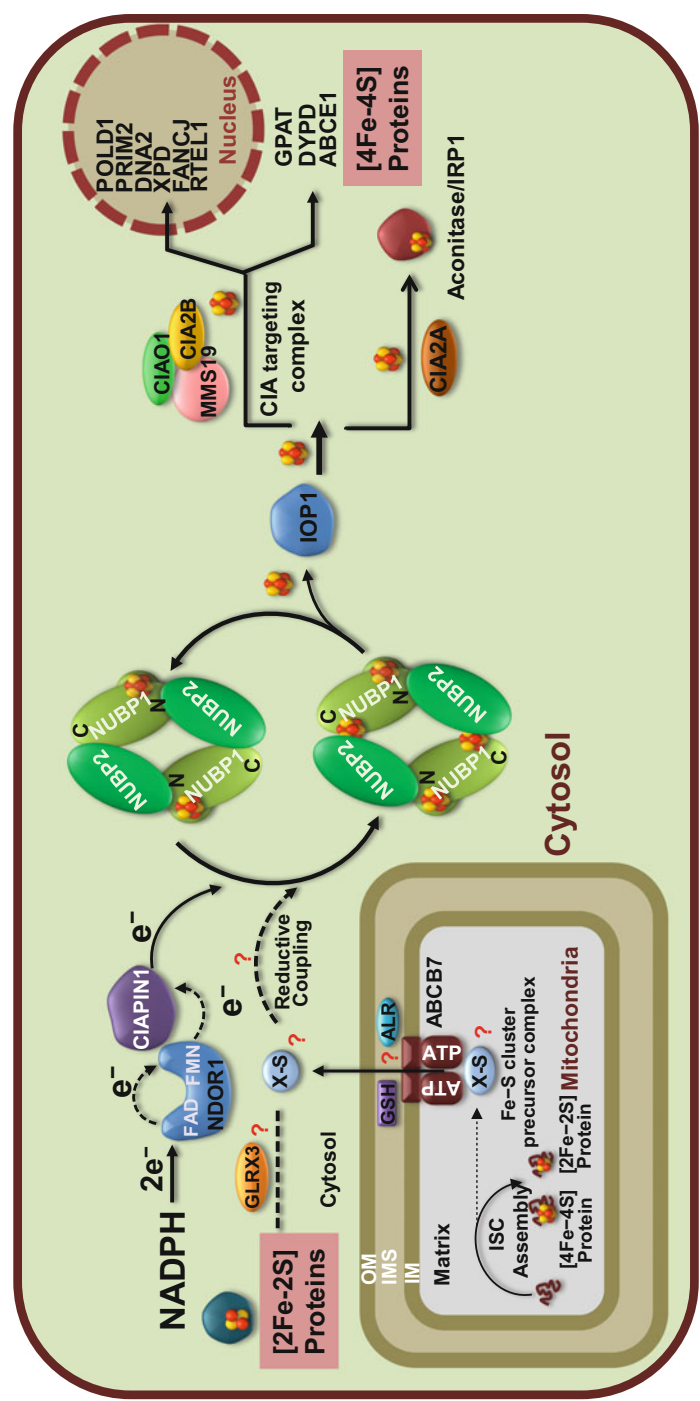


**Fig. 5** Outline of human mitochondrial Fe-S cluster biogenesis. In human, NFS1-ISD11 protein, complex serves as the sulfur donor protein, whereas frataxin is the putative iron donor. Ferredoxin reductase (FDXR) transfers an electron from NADPH to the scaffold protein ISCU via ferredoxin 2 (FDX2). ISCU serves as a platform for the assembly of Fe-S cluster. The transfer of the assembled cluster is assisted by mtHsp70 protein HSPA9, J-protein HSCB; nucleotide exchange factor GRPEL1 and monothiol glutaredoxin GLRX5. These components are sufficient for assembly of [2Fe-2S] proteins. Additional ISC targeting factors involve in the assembly of [4Fe-4S] proteins. ISCA1, ISCA2, and IBA57 along with other additional protein factors allow the formation of [4Fe-4S] cluster and their target specific insertion into mitochondrial apo-proteins. NUF1, which transiently binds to [4Fe-4S] cluster and BOLA3 participate in the maturation of complex I, complex II, and lipoic acid synthase (LIAS). The P-loop NTPase NUBPL which also binds to transferable [4Fe-4S] plays an important role in the incorporation of Fe-S cluster, specifically in complex I. The precise mechanism of [4Fe-4S] cluster maturation is still unclear

### 6.1.7 Maturation of [4Fe-4S] Clusters

The release of Fe-S cluster from scaffold protein may be directly or indirectly used for maturation of [2Fe-2S] proteins, but syntheses of [4Fe-4S] clusters require additional protein factors. In human, three major proteins ISCA1 (Isa1 in yeast), ISCA2 (Isa2 in yeast), and folate-binding protein IBA57 (Iba57 in yeast) play a pivotal role in conversion of [2Fe-2S] clusters to [4Fe-4S] clusters (Fig. 5) (Gelling et al. 2008; Muhlenhoff et al. 2011; Sheftel et al. 2012). Studies conducted in yeast model system reveal that these three proteins (Isa1/Isa2/Iba57) physically interact with each other probably involving in the same biochemical step by forming a complex (Gelling et al. 2008). A similar homologous function for these proteins is assigned in humans. Depletion of ISCA1, ISCA2, or IBA57 in human cell lines results in enormously swollen and enlarged mitochondria, which virtually lack cristae-like membranes, thereby signifying the importance of these proteins in the mitochondrial biogenesis. Moreover, the activities of mitochondrial [4Fe-4S] cluster harboring proteins, including respiratory complex I, complex II, aconitase and lipoic acid synthase (LIAS) were highly compromised in depleted cells (Sheftel et al. 2012; Song et al. 2009). However, the activity of [2Fe-2S] cluster containing enzyme ferrochelatase, which participates in the mitochondrial heme biogenesis, remained unaffected, implying that their contribution in the maturation of [2Fe-2S] proteins is limited. Even though ISCA1/2 receives [2Fe-2S] cluster from GLRX5 by a specific protein-protein recognition mechanism, the precise function of ISCA1, ISCA2, and IBA57 proteins in the late phase of [4Fe-4S] cluster maturation is still unknown (Lu et al. 2010).

In addition to ISCA1, ISCA2, and IBA57 proteins, various other specialized proteins have been identified in the late machinery that are responsible for trafficking the clusters or help in the maturation of specific Fe-S cluster target proteins (Fig. 6). Human mitochondrial NFU1 (Nfu1 in yeast), which was previously believed to be an alternative scaffold like ISCU protein plays a vital role in [4Fe-4S] cluster maturation during late phase of cluster targeting (Tong et al. 2003). Studies conducted in patients with NFU1 deficiency characterized this protein as a late cluster targeting factor, which specifically participates in the maturation of [4Fe-4S] cluster harboring subunits of respiratory complex I, complex II, and lipoic acid synthase (LIAS). However, NFU1 is not required for the maturation of aconitase or [2Fe-2S] cluster proteins (Tong et al. 2003; Navarro-Sastre et al. 2011). The second dedicated targeting factor for the maturation of specified subsets of [4Fe-4S] protein is BOLA3, which belongs to BolA family (Aim1 in yeast). Studies in patients with BOLA3 mutation showed that deficiency of the protein leads to defective maturation of complex I, complex II, and LIAS, which is similar to NFU1 deficiency, suggesting that BOLA3 might function along with NFU1 (Li and Outten 2012; Melber et al. 2016). In humans, BolA family consists of two other protein members, BOLA1 and BOLA2. Until date, role of BOLA1 and BOLA2 in [4Fe-4S] protein maturation is not clearly established (Willems et al. 2013; Zhou et al. 2008).



**Fig. 6** Tentative model of mitochondrial ISC transport and biogenesis of extra-mitochondrial Fe-S clusters. Biogenesis of cytosolic and nuclear Fe-S cluster requires the mitochondrial ISC assembly machinery and the mitochondrial ISC transport system. Mitochondria transfer an unknown-compound with a sulfur moiety (X-S) to cytosol via the ABC transporter ABCB7 with assistance from sulphydryl oxidase protein ALR and glutathione (GSH). The unknown-compound can directly be used as a precursor of cytosolic [2Fe-2S] proteins and cytosolic GLRX3 might play an important role to target the cluster to an apo-protein, considering the similar role of mitochondrial GLRX5 in [2Fe-2S] cluster transfer. An electron transport chain is identified in cytosol consisting of NADPH, FAD-FMN co-factor containing protein NDOR1 and CIAPIN1 that transfer the electron for [4Fe-4S] cluster assembly, probably for reductive

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**Fig. 6** (continued) coupling of two [2Fe-2S] clusters. Assembly of the cytosolic [4Fe-4S] clusters is executed in a hetero-tetrameric protein scaffold consists of NUBP1 and NUBP2 proteins, which transfer the assembled [4Fe-4S] cluster to an intermediate [4Fe-4S] carrier IOP1. The late CIA targeting tri-protein complex which consists of CIAO1, CIA2B, and MMS19 transfers the [4Fe-4S] cluster for maturation of dedicated nuclear and cytosolic proteins like DNA polymerase  $\delta$  (POLD1), DNA primase large subunit (PRIM2), DNA replication ATP-dependent helicase/nuclease 2 (DNA2), xeroderma pigmentosum protein D (XPD), Fanconi anemia protein J (FANCI), regulator of telomere elongation 1 (RTEL1), glutamine phosphoribosyl-pyrophosphate amidotransferase (GPAT), dihydropyrimidine dehydrogenase (DPYD), and ATP-binding cassette E sub-family protein 1 (ABCE1). On the other hand, CIA2A protein involves in the maturation of cytosolic aconitase/iron regulatory protein 1 (IRP1)



The third dedicated targeting factor which specifically involves in the maturation of [4Fe-4S] cluster containing subunits of complex I is a P-loop NTPase protein, NUBPL (also known as IND1), which can bind to a Fe-S cluster transiently via a conserved CXXC motif (Sheftel et al. 2009). Studies in yeast (*Yarrowia lipolytica*), plants, and humans show that deficiency of Ind1 leads to low levels of Fe-S cluster containing subunits of complex I, whereas levels of other mitochondrial [4Fe-4S] proteins remain unaltered, suggesting that NUBPL specifically participates in complex I maturation. Depletion of NUBPL in human cell lines demonstrates a decreased level of several subunits (NDUFS1, NDUFV1, NDUFS3, and NDUFA13) of the peripheral arm of complex I (Sheftel et al. 2009). Moreover, patients with NUBPL mutation harbor a defective complex I, further confirming the importance of NUBPL in complex I maturation (Kevelam et al. 2013; Calvo et al. 2010). Importantly, *Saccharomyces cerevisiae* does not have an Ind1 ortholog since it lacks a typical respiratory complex I (Bych et al. 2008; Sheftel et al. 2009). Although different studies have demonstrated that a distinct set of protein factors are needed for maturation of [4Fe-4S] clusters, a clearer picture of the maturation process remains to be illustrated.

## 6.2 Biogenesis of Extra-Mitochondrial Fe-S Clusters

Biogenesis of cytosolic and nuclear Fe-S cluster is an intricate and complex process in higher eukaryotes. Though it has been examined partially in the yeast model system, in human extra mitochondrial Fe-S cluster biogenesis machinery is minimally characterized. In continuum, presence of a well-coordinated cytosolic Fe-S cluster assembly (CIA) machinery has been identified in higher eukaryotes that perform the biogenesis and maturation of cytosolic and nuclear Fe-S clusters. Notably, the maturation of the cytosolic and nuclear Fe-S protein is strictly dependent on the mitochondrial ISC assembly machinery (Kispal et al. 1999; Gerber et al. 2004) and mitochondrial ISC export system. However, the molecular details of this dependence are not well characterized.

### 6.2.1 ISC Export System

It is believed that mitochondrial ISC assembly machinery synthesizes an unknown compound which is transported from the mitochondrial matrix to cytosol and thereafter CIA machinery participates in the synthesis of cytosolic and nuclear Fe-S proteins. As the mitochondrial sulfur delivery by the sulfur donor protein, NFS1 is essential for the synthesis of both mitochondrial and cytosolic Fe-S cluster biogenesis, therefore it is predicted that the unknown compound is a sulfur containing moiety (X-S) (Biederbick et al. 2006). On the other hand, whether iron is also exported as a part of the unknown compound or supplied from cytosol is not well defined. The other major contributor of the CIA machinery apart from



mitochondrial ISC assembly machinery is the mitochondrial export system. In human, the export reaction is mediated by ABC transporter ABCB7 (Atm1 in yeast) which is present in the inner mitochondrial membrane (Biederbick et al. 2006; Pondarre et al. 2006; Cavadini et al. 2007). The human ABCB7 is an adenosine triphosphate-binding cassette transporter superfamily protein, which assembles as homo-dimeric complexes. Depletion of ABCB7 in human cell lines results in increased mitochondrial iron load and decreased activity of cytosolic Fe-S cluster containing enzymes. Importantly, the accumulated iron inside mitochondria is not readily available for the heme synthesis suggesting that iron might be in a complex with sulfur as a unknown compound, which is transported from mitochondria to cytosol as a precursor for the synthesis of cytosolic Fe-S cluster biogenesis (Lange et al. 2001). Additionally, the export process is assisted by the tri-peptide glutathione (GSH) and the sulfhydryl oxidase ALR (also known as GFER), human ortholog of yeast Erv1 present in the inter-membrane space of mitochondria (Fig. 6) (Mesecke et al. 2005). ALR/Erv1 is an FAD-dependent sulfhydryl oxidase that catalyzes disulfide bond formation during MIA40 chaperone mediated import and folding of low molecular weight cysteine-containing proteins in the mitochondrial inter-membrane space (Allen et al. 2005; Banci et al. 2012). Studies in the yeast system shown that ALR/Erv1 plays a major role in maturation of cytosolic Fe-S clusters, since a temperature sensitive mutant of Erv1 exhibits impairment in the activity of cytosolic Fe-S cluster containing enzymes, but not from the mitochondrial counterparts (Lange et al. 2001; Ozer et al. 2015). Similarly, deletion of the *GSH1* gene in yeast which encodes the enzyme that catalyzes the first-step of GSH biosynthesis results in a substantial decrease in cytosolic Fe-S protein maturation while the activity of mitochondrial Fe-S proteins remains unaltered (Sipos et al. 2002). Recent in vitro studies provide evidence that GSH coordinates with [2Fe-2S] cluster via the cysteine residue present on GSH as [2Fe-2S] (GSH)<sub>4</sub><sup>2-</sup> and transported to the cytosol by Atm1p (Li and Cowan 2015). However, precise chemical nature of this species which transports to the cytosol is to be further confirmed, in vivo. In short, maturation of cytosolic Fe-S proteins in human or in yeast is strictly reliant on the mitochondrial ISC assembly machinery and ISC export system and probably supply of pre-assembled [2Fe-2S] clusters for the biogenesis of extra mitochondrial Fe-S proteins.

### 6.2.2 Assembly of Extra-Mitochondrial Fe-S Clusters

Studies performed in human cell lines and yeast model have provided the preliminary evidence for the presence of minor amount of cytosolic isoform of some of the mitochondrial ISC assembly proteins (Tong and Rouault 2000; Condo et al. 2006, 2010; Acquaviva et al. 2005). However, their specific role in the cytosolic Fe-S protein maturation or in some other process is not unveiled. Apart from these cytosolic isoform of mitochondrial ISC assembly proteins, nine cytosolic proteins have been identified, which actively participate in the maturation of cytosolic and nuclear Fe-S proteins.

The source of sulfur donor for cytosolic Fe-S cluster biogenesis is not firmly confirmed. It is believed that sulfur transports out of the mitochondria as a part of Fe-S cluster precursor complex which is readily utilized for the synthesis of extra-mitochondrial Fe-S clusters. In accordance with the sulfur donation process, there is no specific iron donor reported in cytosol. Presumably, iron also transported as a part of the same Fe-S cluster precursor complex from the mitochondria. In human, one dedicated electron transport chain system has been reported in the cytosol which delivers electrons for the maturation of extra-mitochondrial Fe-S protein. The electron transfer chain system of the human cytosolic machinery consists of NADPH, FAD and FMN containing protein, NDOR1 (Tah18 in yeast) and Fe-S cluster harboring protein CIAPIN1 (Dre2 in yeast) (Banci et al. 2013; Netz et al. 2010). Findings in yeast homologue proteins indicate that the electrons are supplied from NADPH to [2Fe-2S] center of Dre2 via the FAD and FMN centers of Tah18 (Fig. 6). The precise role and specific destination of electrons during cytosolic Fe-S protein maturation is not well understood. Importantly, absence of Dre2 and Tah18 results in loss of [4Fe-4S] cluster maturation on the cytosolic scaffold protein implying the necessity of the electron transport system for cluster maturation (Vernis et al. 2009; Netz et al. 2012; Paul and Lill 2015). Considering the recent proposal that mitochondria export [2Fe-2S] clusters for extra-mitochondrial cluster synthesis, the electrons might be used for reductive coupling of [2Fe-2S] clusters to generate [4Fe-4S] clusters on the cytosolic scaffolds. However, there is no experimental evidence established for this hypothesis.

The function of cytosolic scaffold protein is executed by the P-loop NTPase proteins NUBP1 (Nbp35 in yeast) and NUBP2 (Cfd1 in yeast). These two proteins share a high degree of sequence similarity and constitute a hetero-tetrameric protein complex. Investigation in the yeast model revealed that the hetero-tetrameric complex coordinates with four [4Fe-4S] clusters. The N-termini of the two Nbp35 of the hetero-tetrameric complex bind tightly to one [4Fe-4S] cluster per monomer through a conserved ferredoxin-like CX<sub>13</sub>CX<sub>2</sub>CX<sub>5</sub>C motif which is essential for Nbp35 function. Each of the other two [4Fe-4S] clusters coordinates weakly to the interface of two different or identical protein monomers with the help of a conserved CX<sub>2</sub>C motif present at the C-terminus of both the proteins. Since the C-terminus [4Fe-4S] clusters are loosely associated in the hetero-tetrameric complex, the clusters can readily be transferred to a target protein after their maturation (Netz et al. 2007, 2012). The presence of cytosolic glutaredoxin, GLRX3 has also been reported in human cells. However, the precise function of the protein in cytosolic Fe-S cluster maturation is unclear. The protein might function as a transient [2Fe-2S] cluster carrier-like mitochondrial GLRX5; however, experimentally this idea is not validated.

### 6.2.3 Transfer and Targeting of Extra-Mitochondrial Fe-S Clusters

The targeting of newly assembled [4Fe-4S] clusters from NUBP1-NUBP2 scaffold complex to the cytosolic and nuclear apo-protein is highly complex and partially

characterized in higher eukaryotes (Fig. 6). The transfer of the assembled cluster is performed by a number of dedicated proteins comprising iron-only hydrogenase-like protein IOP1 (also known as NARFL) (Nar1 in yeast) and CIA targeting complex formed of CIAO1 (Cia1 in yeast), CIA2A (absent in yeast), CIA2B (Cia2 in yeast), and MMS19 (Met18 in yeast). Nonetheless, the detailed molecular function of these proteins and their specific targets during cluster transfer is poorly defined.

Studies in human cell lines demonstrate that depletion of IOP1 causes decreased activity of extra-mitochondrial Fe-S proteins. However, activity of mitochondrial Fe-S proteins remained unaffected suggesting the critical importance of the protein in CIA machinery (Song and Lee 2008, 2011). In yeast, Nar1 has shown the ability to co-ordinate two [4Fe-4S] clusters (Urzica et al. 2009; Balk et al. 2004). Presumably, IOP1 connects the early and late acting CIA protein components for efficient transfer of the cluster to target apo-proteins. Additionally, recent findings propose that human IOP1 also plays a role in the cellular defense against hyperopia-induced oxidative stress (Corbin et al. 2015).

On the other hand, CIAO1, CIA2A (also known as FAM96A), CIA2B (also known as FAM96B), and MMS19 function during the late targeting process of extra-mitochondrial protein maturation. CIAO1 associates with either CIA2A or CIA2B and MMS19 for targeting Fe-S clusters to client proteins (Fig. 6) (Stehling et al. 2012; Gari et al. 2012). The CIAO1-CIA2B-MMS19 targeting complex involves in maturation of most of the cytosolic-nuclear Fe-S proteins. For example, the tri-protein complex delivers cluster to a number of cytosolic proteins that participate in nucleotide metabolism [glutamine phosphoribosyl-pyrophosphate amidotransferase (GPAT) and dihydropyrimidine dehydrogenase (DPYD)] and translation initiation and termination [ATP-binding cassette E sub-family protein 1 (ABCE1)]. The same tri-protein complex involves in maturation of nuclear proteins that participate in DNA replication [DNA polymerase  $\delta$  (POLD1), DNA primase large subunit (PRIM2) and DNA replication ATP-dependent helicase/nuclease 2 (DNA2)], DNA repair [xeroderma pigmentosum protein D (XPD) and Fanconi anemia protein J (FANCF)] and regulation of telomere length [Regulator of Telomere Elongation 1 (RTEL1)]. Notably, maturation of GPAT and POLD1 is not affected by the absence of MMS19 or CIA2B respectively, suggesting the dispensable nature of these two CIA targeting factors for maturation of specific Fe-S proteins. Besides, CIA2A protein is specific for maturation of cytosolic aconitase/iron regulatory protein 1 (IRP1) and stabilizes iron regulatory protein 2 (IRP2), thereby contributes to the maintenance of iron homeostasis in cell (Stehling et al. 2013). In accordance with the targeting of [4Fe-4S] clusters to a bonafide apo-protein, the [2Fe-2S] cluster transfer to a dedicated cytosolic protein is not known. However, cytosolic GLRX3 might play an importance role in targeting [2Fe-2S] clusters to the client protein after the export from mitochondria.

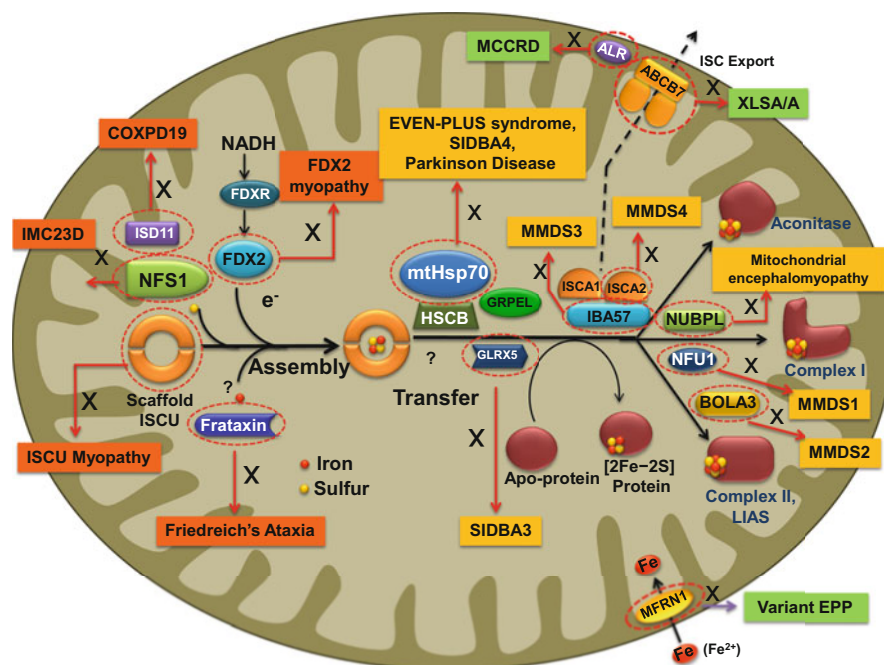
## 7 The Importance of Fe-S Cluster Biogenesis: Implications in Human Health and Disease

Iron-sulfur (Fe-S) clusters are fundamental constituents of a cell and play a pivotal role in cellular function and survival. Activity of a wide range of proteins and enzymes inside a cell is dependent upon the physical integration of Fe-S cluster into their protein backbone as a co-factor. Thereby, it is fairly obvious that impairment of Fe-S cluster biogenesis process will cause pathological conditions in human (Fig. 7). Mutation in genes of the core Fe-S cluster biogenesis pathway as well as in targeting factors and transport machinery leads to development of various diseases with a wide range of pathological symptoms (Lill and Muhlenhoff 2008; Rouault and Tong 2008; Beilschmidt and Puccio 2014; Stehling et al. 2014; Rouault 2012; Ye and Rouault 2010). Most of the diseases are fatal, notably, during early childhood. The following are the major diseases that are associated with a deficiency of the core Fe-S cluster assembly components, Fe-S cluster targeting factors, and transport system.

### 7.1 Diseases Associated with Core Fe-S Cluster Assembly Machinery Impairment

Defects in the core biogenesis machinery which is constituted of NFS1, ISD11, Frataxin, ISCU, FDXR and FDX2 proteins cause severe pathological conditions. Friedreich's ataxia (FRDA) (OMIM #229300) (Online Mendelian Inheritance in Man, OMIM) is the most common among the diseases linked to malfunctioning of Fe-S cluster biosynthesis and is caused by the deficiency or loss of function of Frataxin. This neurodegenerative disorder is characterized by progressive gait, limb ataxia, cardiomyopathy, and diabetes mellitus. The majority (>95%) of patients with FRDA are homozygous for large expansions of a GAA triplet repeat sequence (66–1,800 triplets) located within the first intron of the *frataxin* gene which results in depleted levels of the protein. A minor number of patients are compound heterozygotes for the GAA expansion in one allele and point mutation(s) in the other *frataxin* allele (112–116).

The second most prevalent disease after Friedreich's ataxia is ISCU myopathy (OMIM #255125) which is characterized by lifelong exercise intolerance, where minor exertion causes pain of active muscles, shortness of breath, fatigue, and tachycardia. The disease is caused by loss of function of the Fe-S cluster scaffold protein ISCU. Most affected individuals are homozygous for a mutation in intron 4 (g.7044G>C) of ISCU that results in synthesis of aberrantly spliced ISCU mRNA, successively causing accumulation of truncated non-functional ISCU protein. Recently, compound heterozygous patients with severe myopathy have been identified. These patients have a common intronic splice mutation (g.7044G>C) on one allele and a novel missense mutation (c.149G>A) in exon 3 on the other



**Fig. 7** Human mitochondrial disorders linked to defects in Fe-S cluster biogenesis. A large number of human diseases have been reported to be associated with defects in different steps of the Fe-S cluster biogenesis process. The first set of mitochondrial diseases (orange boxes) is associated with defects in the core assembly pathway. Deficiency or loss of function of frataxin (FXN) protein leads to neurodegenerative disorder Friedreich's ataxia. The central scaffold protein ISC is crucial for mitochondrial function as the defect in the protein causes exercise intolerance disorder ISCU myopathy. Defect in sulfur donor protein NFS1 causes Infantile Mitochondrial Complex II/III deficiency (IMC23D). Mutation of sulfur transfer assisting protein ISD11 results in Combined Oxidative Phosphorylation Deficiency 19 (COXPD19). Ferredoxin 2 (FDX2) protein is involved in electron transport and is responsible for FDX2 myopathy. The second set of diseases (yellow boxes) are due to defects in the protein components that involve in cluster transfer and maturation. Deficiency of monothiol glutaredoxin protein GLRX5 that helps in the [2Fe-2S] cluster transfer process leads to sideroblastic anemia 3 (SIDBA3). Mitochondrial Hsp70 protein HSPA9 which assists the transfer process is a crucial component as different mutations in the protein cause distinct types of mitochondrial disorders, namely Parkinson disease (PD), EVEN-PLUS syndrome, and sideroblastic anemia 4 (SIDBA4). Decreased levels of NUBPL protein, which plays an important role in transfer of Fe-S cluster to complex I lead to development of mitochondrial encephalomyopathy. NUF1 and BOLA3 proteins play a crucial role in maturation of [4Fe-4S] cluster in complex I, II, and lipoic acid synthase (LIAS) proteins, and specific mutations within the genes of *NUF1* and *BOLA3* result in Mitochondrial Dysfunction Syndrome 1 (MMDS1) and Mitochondrial Dysfunction Syndrome 2 (MMDS2), respectively. Loss of function of IBA57 protein which involves in general maturation of [4Fe-4S] cluster proteins results in Multiple Mitochondrial Dysfunction Syndrome 3 (MMDS3). Moreover, mutation in the [4Fe-4S] cluster targeting factor ISCA2 causes Multiple Mitochondrial Dysfunction Syndrome 4 (MMDS4). The last set of diseases (green boxes) is related to transport defects. Defect in synthesis of mitochondrial iron import protein, mitoferrin 1 (MFRN1) causes variant erythropoietic protoporphyria. Lastly, impaired function of component of the mitochondrial ISC export machinery ABCB7 leads to X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A)

allele which changes a conserved glycine residue to a glutamate at the 50th position (p.G50E) (Kollberg et al. 2009; Nordin et al. 2011, 2012; Olsson et al. 2008).

Apart from ISCU myopathy, FDX2 myopathy is a second type of isolated muscle myopathy caused by deficiency of Ferredoxin 2 (FDX2) that transfers electrons to scaffold protein from NADPH via FDXR for Fe-S cluster biogenesis. This disease is characterized by myopathy and lactic acidosis along with compromised ETC complex activity, in muscles. Homozygous missense mutation in the first codon of the *FDX1L* gene, encoding the mitochondrial FDX2 protein leads to development of FDX2 myopathy (Fig. 7). The mutation disrupts the ATG translation initiation site resulting in conversion of first methionine residue to leucine (p.M1L) which leads to undetectable protein levels in muscle and fibroblasts (Spiegel et al. 2014; Sheftel et al. 2010).

The fourth critical disease associated with core Fe-S cluster biogenesis machinery is infantile mitochondrial complex II/III deficiency (IMC23D) which is a recently identified fatal autosomal recessive disease caused by impaired function of sulfur donor protein NFS1. Exome sequencing and autozygosity mapping reveal that a homozygous missense mutation c.215G>A converts 72nd arginine residue of NFS1 to glutamine (p.R72Q), hence leads to the development of IMC23D. This disease is characterized by lactic acidosis, hypotonia, deficiency of complex II and III of ETC, multisystem organ failure, and abnormal mitochondrial morphology (Farhan et al. 2014).

Lastly, loss of function of the mitochondrial matrix protein ISD11 which assists the function of the sulfur donor protein NFS1 results in respiratory distress disorder combined oxidative phosphorylation deficiency 19 (COXPD19) (Fig. 7). The affected individuals are homozygous for a missense mutation (c.203G>T) in the *LYRM4* gene on chromosome 6p25 which codes the ISD11 protein, resulting in the conversion of a conserved 68th arginine residue to leucine (p.R68L). This disease is characterized by respiratory distress, hypotonia, gastroesophageal reflux, hepatomegaly, and severe lactic acidosis in neonates. Studies on patient liver and muscle samples have demonstrated decreased activities of mitochondrial respiratory complexes. Post-mortem examination of COXPD19 patients has shown widened fiber size in skeletal muscle, increased lipid content in muscle and liver along with the presence of abnormal mitochondria (Lim et al. 2013).

## 7.2 Diseases Associated with Impairment of Cluster Targeting and Maturation Machinery

The transfer of [2Fe-2S] clusters and maturation of [4Fe-4S] clusters are intricate and indispensable processes in mammalian mitochondria during Fe-S cluster biogenesis. Until date, seven proteins of the cluster targeting and the maturation pathways have been reported to be associated with human mitochondrial disorders. The central protein of the cluster transfer cycle is mitochondrial Hsp70 protein,



HSPA9 (Gene MIM No \*600548). Notably, human mitochondria harbor only one mtHsp70 that is HSPA9 in contrast to yeast mitochondria. Thereby, apart from transferring Fe-S cluster to client proteins, HSPA9 functions in multiple processes, such as mitochondrial protein import, folding and maintenance of mitochondrial DNA. Because of this multi-functionality of the protein, a given HSPA9 mutant could affect multiple cellular processes, including Fe-S cluster biogenesis. HSPA9 has been reported to be associated with three different types of mitochondrial diseases. A recent study demonstrates that compound heterozygous mutation in *HSPA9* gene, c.383A>G (p.Y128C) mutation in one allele and c.882\_883delAG (p.V296\*) in the other, causes a rare EVEN-PLUS syndrome with congenital malformations and skeletal dysplasia. Additionally, a homozygous mutation c.376C>T (p.R126W) of HSPA9 has also been observed to be associated with EVEN-PLUS syndrome (Phenotype MIM No. #616854) (Royer-Bertrand et al. 2015). Another study suggests that specific mutations in the HSPA9 protein lead to congenital sideroblastic anemia 4 (SIDBA4) (Phenotype MIM No. #182170) which is characterized by the presence of ring sideroblasts in the bone marrow as a result of pathological mitochondrial iron deposits (Schmitz-Abe et al. 2015). Lastly, two heterozygous point mutants of HSPA9, p.R126W and p.P509S have been reported to be associated with Parkinson disease. Importantly, the role of these different diseases associated mutant variants in Fe-S cluster biogenesis impairment has not been proven experimentally. However, considering the immense importance of HSPA9 during Fe-S cluster transfer process, the Fe-S cluster maturation process would be affected since human mitochondria harbors only one mtHsp70 dedicated for different functions (Wadhwa et al. 2015; Goswami et al. 2012; Jin et al. 2006).

The second important protein required for [2Fe-2S] cluster transfer is GLRX5 and deficiency of the protein causes sideroblastic anemia 3 (SIDBA3) (Phenotype MIM No. #616860) (Fig. 7). A homozygous (c.294A>G) mutation in the *GLRX5* gene which interferes with intron 1 splicing and successively reduces *GLRX5* mRNA levels drastically results in the disease manifestation (Camaschella et al. 2007). Recently, a compound heterozygous Chinese patient, for two missense mutations (p.K101Q and p.L148S) in his *GLRX5* gene has been reported to be affected with congenital sideroblastic anemia (Liu et al. 2014). Apart from sideroblastic anemia, spasticity with hyperglycinemia (SPAHC) (Phenotype MIM No. #616859) has been observed to be associated with homozygous mutation (deletion c.151\_153delAAG) in *GLRX5* gene (p.K51del) with a childhood-onset of spastic paraplegia, spinal lesion, and optic atrophy (Baker et al. 2014).

Following Friedreich's ataxia and ISCU myopathy, NFU1 associated multiple mitochondrial dysfunction syndrome 1 (MMSD1) (Phenotype MIM No. #605711) is the third most prevalent disease related to Fe-S cluster biogenesis defect which is characterized by lack of neurological development, lactic acidosis, respiratory failure, weakness, and early death. A homozygous missense mutation (c.545G>A) in the *NFU1* gene results in p.R182Q substitution causing MMSD1 with undetectable amounts of mature protein in fibroblast mitochondria (Cameron et al. 2011). Moreover, various other studies have reported several additional mutations in the NFU1 protein (p.G208C, p.C210F and p.G189R) that result in MMSD1. These patients have

compromised ETC complex activity and a defective lipoic acid biosynthesis process (Navarro-Sastre et al. 2011; Nizon et al. 2014; Invernizzi et al. 2014; Ferrer-Cortes et al. 2012). Recently, patients with early onset of lethal spastic paraplegia have also been reported to be associated with *NFU1* compound heterozygous mutation (p. P49LfsTer8 and p.G189R) (Tonduti et al. 2015). In accordance with MMDS1, homozygous missense mutation in *BOLA3* gene causes multiple mitochondrial dysfunctions syndrome 2 (MMDS2) with associated hyperglycinemia (Phenotype MIM No. #614299). Interestingly, MMDS2 demonstrates clinical and biochemical phenotypes similar to *NFU1* deficiency (Navarro-Sastre et al. 2011). Several types of mutations in *BOLA3* gene have been found in patients with MMDS2 which lead to dilated cardiomyopathy, severe epileptic encephalopathy, and optic atrophy with reduced mitochondrial respiration (Maio and Rouault 2015). Among the reported *BOLA3* mutations, homozygous c.136C>T point mutation in exon 2 results in a truncated *BOLA3* protein (p.R46\* stop codon) with less severe MMDS2 phenotypes. However, homozygous single base pair duplication (c.123dupA) within *BOLA3* gene causes a frame shift with a premature translation stop (p.E42RfsTer13) and homozygous missense mutation (c.200T>A) on exon 3 results in p.I67N substitution leading to severe MMDS2 phenotypes (Maio and Rouault 2015). The fifth important protein in the cluster targeting and maturation machinery associated disease category is *IBA57*, which causes multiple mitochondrial syndrome 3 (MMDS3) (Phenotype MIM No. #615330) with severe myopathy and encephalopathy. A homozygous missense mutation in the *IBA57* gene, c.941A>C (p.Q314P) results in manifestation of MMDS3. The levels of *IBA57* protein in the skeletal muscle and cultured skin fibroblasts of the affected individuals were observed to be severely depleted in p. Q314P substitution (Ajit Bolar et al. 2013). A recent report suggests that a homozygous c.436C>T (p.R146W) mutation in the *IBA57* gene causes fatal infantile leukodystrophy along with neurodegeneration (Debray et al. 2015). In addition to the aforementioned disease conditions, patients with hereditary spastic paraplegia along with optic atrophy and peripheral neuropathy have been identified. These patients have a homozygous donor splice-site mutation in the *IBA57* gene which leads to severely decreased *IBA57* mRNA levels or had aberrantly spliced *IBA57* mRNA with a premature stop codon (Lossos et al. 2015). In addition to MMDS3, a recent report suggests that homoallelic c.229G>A (p.G77S) mutation in the *ISCA2* gene leads to infantile neurodegenerative mitochondrial disorder also referred to as multiple mitochondrial syndrome 4 (MMDS4) (Phenotype MIM No. #616370) causing leukodystrophy and neuroregression (Al-Hassnan et al. 2015).

Lastly, mutations in the *NUBPL* gene result in mitochondrial encephalomyopathy which is characterized by deficiency in activity of complex I (Phenotype MIM No. #252010) of the ETC complex. Most of these patients are compound heterozygous for a null mutation in one allele and the other allele carries one branch site mutation (c.815-27T>C) along with one c.166G>A missense mutation. The branch site mutation causes synthesis of two splice variants along with the wild-type transcript resulting in low levels of *NUBPL* protein (Tucker et al. 2012; Kevelam et al. 2013; Wydro and Balk 2013).



### 7.3 Diseases Associated with Import and Transport Machinery

The other human mitochondrial diseases associated with Fe-S cluster biogenesis defects are due to mutations in the import and transport proteins. Mitochondrial iron import which is primarily mediated by solute carrier mitoferrin1 (also known as MFRN1) (Gene MIM No \*610387) is crucial for Fe-S protein biogenesis and heme formation. Although MFRN1 does not involve directly in classical Fe-S cluster biogenesis pathway, yet it boosts the process by importing iron as a raw material. Erythropoietic porphyria (EPP) is a disease caused by the absence of Fe-S cluster containing enzyme ferrochelatase which results in accumulation of protoporphyrin IX leading to photosensitivity of the skin and liver damage. Furthermore, a variant form of EPP has been identified with aberrant splicing of iron importer MFRN1 mRNA which is characterized by decreased ferrochelatase activity and similar clinical symptoms. Transcriptome analysis of patients with variant EPP demonstrates that almost 50% of the primary transcript of MFRN1 is improperly processed causing degradation of the transcripts or synthesis of nonfunctional truncated protein (Wang et al. 2011).

The second disease in this category is X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A) (Phenotype MIM No. #301310) which is another example of a rare congenital sideroblastic anemia apart from GLRX5 and HSPA9 deficiency associated sideroblastic anemia (Fig. 7). The disease is caused by defects in the mitochondrial ISC export machinery component, ABCB7. Missense mutation in the *ABCB7* gene mapped to the X-chromosome causes XLSA/A which is a recessively inherited disorder and characterized by an early childhood onset of cerebellar atrophy, mild anemia with microcytosis, and hypochromia. Since ABCB7 participates in ISC export, XLSA/A results in iron loaded mitochondria (sideroblasts) and defective biogenesis of cytosolic Fe-S proteins (Bekri et al. 2000; Maio and Rouault 2015). Lastly, a homozygous mutation in the mitochondrial intermembrane space (IMS) disulfide relay system protein GFER results in myopathy with cataract and combined respiratory-chain deficiency (MCCRD) (Phenotype MIM No. #613076). Additionally, the patients have sensorineural hearing loss, developmental delay with abnormal ultra-structural morphology of the mitochondria and enlarged IMS. The c.581G>A missense mutation in exon 3 of *GFER* gene, resulting in a p.R194H substitution leads to manifestation of MCCRD (Di Fonzo et al. 2009).

## 8 Concluding Remarks

In recent times a sound knowledge of the biogenesis of Fe-S cluster claims a crucial importance in modern biology considering the close association of human health and disease with Fe-S cluster biogenesis defects. Although the basic concept of

Fe-S cluster biogenesis has been established satisfactorily, there are several aspects of the biogenesis and maturation process that need to be examined meticulously. For instance, the source of iron for mitochondrial Fe-S cluster assembly process still remains ambiguous. On the other hand, molecular chemistry of the initial assembly process, a detailed role of cluster targeting factors in targeting and maturation process and mechanism of Fe-S cluster complex export from mitochondria to cytosol has to be still uncovered. Biogenesis and assembly of cytosolic and nuclear Fe-S proteins still require meticulous investigation to obtain a clearer picture. According to the present knowledge about the ISC export system of mitochondria, probably [2Fe-2S] cluster readily comes from mitochondria, which can be directly utilized for [2Fe-2S] proteins, whereas [4Fe-4S] cluster is synthesized in cytosol by reductive coupling of two [2Fe-2S] clusters in the presence of an electron transfer system and other accessory protein partners. At the same time, significance of cytosolic isoforms of mitochondrial Fe-S cluster proteins like sulfur donor NFS1 or scaffold protein ISCU requires further investigation.

In spite of the immense research that has been performed in the field of Fe-S cluster biogenesis, one distinct picture of the multi protein complexes formed during biogenesis process step by step has not been illustrated so far. Although three-dimensional structures of several Fe-S cluster biogenesis proteins have been solved from various species, further development in Fe-S cluster biogenesis study demands three-dimensional structure of holo-form of scaffold protein in association with other partner proteins involved throughout the biogenesis process. This may aid in understanding the molecular mechanism of de novo Fe-S cluster assembly precisely.

Along with the development and remarkable progress in the field of Fe-S cluster biogenesis, the number of reports of various mitochondrial disorders associated with Fe-S cluster biogenesis impairment has been increasing at an alarming rate. Therefore, there is an urgent need for novel mitochondrial therapeutics to combat these diseases. Since, mitochondrial diseases demonstrate a wide variety of clinical symptoms, thereby identifying the specific cause of the disease becomes very difficult and requires mitoxome sequencing. As a consequence, treatment of mitochondrial disorders or designing an appropriate therapeutic approach for the prevention of mitochondrial diseases turns out to be challenging. On a positive context, widespread research is being carried out on different aspects of combating mitochondrial disorders associated with Fe-S biogenesis, like activation of mitochondrial biogenesis and mitochondrial gene replacement therapy, which gives a strong hope for treatment of affected individuals in the near future.

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# Neurotrophin Trk Receptors: New Targets for Cancer Therapy



Jacopo Meldolesi

**Abstract** In the last few years, exciting reports have emerged regarding the role of the two types of neurotrophin receptors, p75<sup>NTR</sup> and Trks, not only in neurons, where they were discovered, but also in non-neural cells and, especially, in numerous cancers, including breast, lung, colon-rectum, pancreas, prostate, glioblastoma, neuroblastoma, myeloma, and lymphoid tumors. Traditionally, p75<sup>NTR</sup>, activated by all neurotrophins and their precursors, is an inhibitor. In various cancers, however, activated p75<sup>NTR</sup> induces variable effects, from inhibition to stimulation of cell proliferation, dependent on their direct or coordinate/indirect mechanism(s) of action. TrkA, TrkB, and TrkC, activated by distinct neurotrophins, are high affinity stimulatory receptors. In cancers, activation of Trks, especially of TrkB, are stimulators of cell proliferation, aggressiveness, and metastases. In rare cancers, these processes are due not to receptor activation but to fusion or mutation of the encoding genes. A considerable panel of anti-Trk drugs, developed recently, has been investigated both in vitro and in living mice for their effects on cancer cells. Many such drugs protect from cancers by preventing cell proliferation and inducing apoptosis. At present, these drugs are under control by trials, to promote introduction in human therapy. Moreover, anti-Trk drugs have been employed also in combination with classical chemotherapeutic drugs. So far, studies in mice have been positive. The chemotherapeutic/anti-receptor combinations exhibited in fact increased potency and down-regulation of resistance, with no increase of side effects.

**Keywords** Anti-Trk drugs • Anti-Trk/chemotherapeutic combination • Cell proliferation • Direct/indirect mechanisms • Drug trials • Metastasis • NTRK gene fusion • p75<sup>NTR</sup> receptor • Trk receptors

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## 1 Introduction

Two types of neurotrophin (NT) receptors, p75 neurotrophin receptor (p75<sup>NTR</sup>) and Tropomyosin Receptor Kinases (Trks), are known since three decades. The receptor of the first type, a non-enzymatic, trans-membrane protein structurally analogous to the tumor necrosis receptor family members, is a low affinity, mostly inhibitory, multifunctional receptor activated by all NTs, i.e. it is a pan-NT receptor. Trks are three, high affinity, stimulatory tyrosine kinase receptors preferentially activated by specific NTs: NGF for TrkA, BDNF/NT4 for TrkB, and NT3 for TrkC. All Trk receptors operate mostly for cell survival, growth, and development.

Initially, NT receptors were believed to be expressed only by neurons of the central and peripheral nervous systems, where they play critical roles in physiological processes and neurodegenerative diseases. Further studies revealed NT receptors to be expressed also by several, non-neural cell types including astrocytes, oligodendrocytes, macrophages, endocrine cells, immune cells, smooth and striated muscle fibers (Meldolesi 2017; Reichardt 2006).

The importance of NT receptors in cancer cells was discovered initially in gliomas and neuroblastomas. At present, however, it is clear that numerous other cancers, including breast, the various types of lung, colon-rectum, pancreas, prostate, liver, as well as myelomas and lymphoid tumors, express high levels and are affected by NT receptors, especially TrkB and also TrkA. The intracellular signals released by the activation of Trks include 3 pathways: from Ras to ERKs; from PI<sub>3</sub>K to AKT and mTORC; from PLC to PKC. The responses induced depend on many of the processes activated in the course of these pathways, including the stimulation of gene expression, differentially triggered by ERKs and AKT. The final responses of cancer cells include processes such as proliferation, aggressiveness, and metastases (Demir et al. 2016; Hondemarck 2012). Intensive results of the same type, independent however on NT binding, are induced, primarily in variously rare cancers, by fusion of NTRK1 and NTRK2, the genes encoding TrkA and TrkB. The effects in these cancers, sustained by the high expression and constitutive activation of the encoded receptors, correspond to those previously attributed to oncogenic NTRKs (Ricciuti et al. 2017; Vaishnavi et al. 2015).



The intense, growing relevance of Trks in a large number of cancer types has stimulated the pharmacological interest for the receptors, now envisaged as candidate targets for growing drug populations. Trk-focused therapies of many cancer cells are therefore investigated, in vitro as well as in vivo, initially in mice and now also in humans. The aim of this review is to report (1) the progress of knowledge about the role and the mechanisms of NT receptors in cancers; and (2) the perspectives of anti-NT receptor agents in anti-cancer therapies.

## 2 NT Receptors in Cancer Cells

In neurons, separate activation of the two types of NT receptors is known to induce mostly conflicting effects: inhibition and death by p75<sup>NTR</sup>; stimulation and survival by Trks. In addition, the two types of receptors operate complexed with each other, with strengthened Trk-type effects (Meldolesi 2017; Reichardt 2006). In cancer cells the effects of NT receptors are more variable. Their knowledge is relevant not only to understand their propoerties, but also for the development of new, promising anti-cancer therapies.

### 2.1 p75<sup>NTR</sup>, the Repressive NT Receptor

Many effects of p75<sup>NTR</sup> on cancer cells are repressive, due to direct actions. The example reported here concerns glioma cells proliferation and invasion inhibited by p75<sup>NTR</sup>. In the same cells, down-regulation of p75<sup>NTR</sup> signaling triggers proliferation due to activation of various kinases (Wang et al. 2015). However, in studies of various cancers including clear renal carcinoma, melanoma and some glioma cells, proliferation and progression are induced also by high p75<sup>NTR</sup> (Alshehri et al. 2017; De la Cruz-Morcillo et al. 2016; Saltari et al. 2016). These processes are due to indirect effects of the receptor. In human glioblastoma and breast cancers, cell proliferation induced by p75<sup>NTR</sup> depends on the ubiquitin-dependent proteolysis of the anti-proliferative p53 proteins, which is stimulated. Conversely, increased levels of p53 following down-regulation of p75<sup>NTR</sup> induce attenuation of cell proliferation (Zhou et al. 2016). In neuroblastomas, a regression by low p75<sup>NTR</sup> is mediated by high expression of p53 together with stimulation of UNC5D, a receptor that induces expression of pro-apoptotic target genes (Zhu et al. 2013). In breast cancer cells with high p75<sup>NTR</sup>, observed death resistance depends on the competition with TRAIL, TNF-related apoptosis inducing ligand (Wilmet et al. 2011). Further survival effects are induced upon proteolytic cleavage of p75<sup>NTR</sup>, with release of either its intracellular or extracellular domain. In these cases the increased cell survival and proliferation depend not on classical p75<sup>NTR</sup> signaling, but on the effects of relased N- and C-terminal fragments of the receptor (Forsyth et al. 2014; Verbeke et al. 2013).

## 2.2 *Trks, the Stimulatory NT Receptors*

The stimulatory effects of Trks are due to two distinct mechanisms, the activation of the receptors by the binding of the specific NTs and the fusion of the encoding receptors, with production of fused receptors. The properties of the cancers governed by the two mechanisms will be presented in the following subsections.

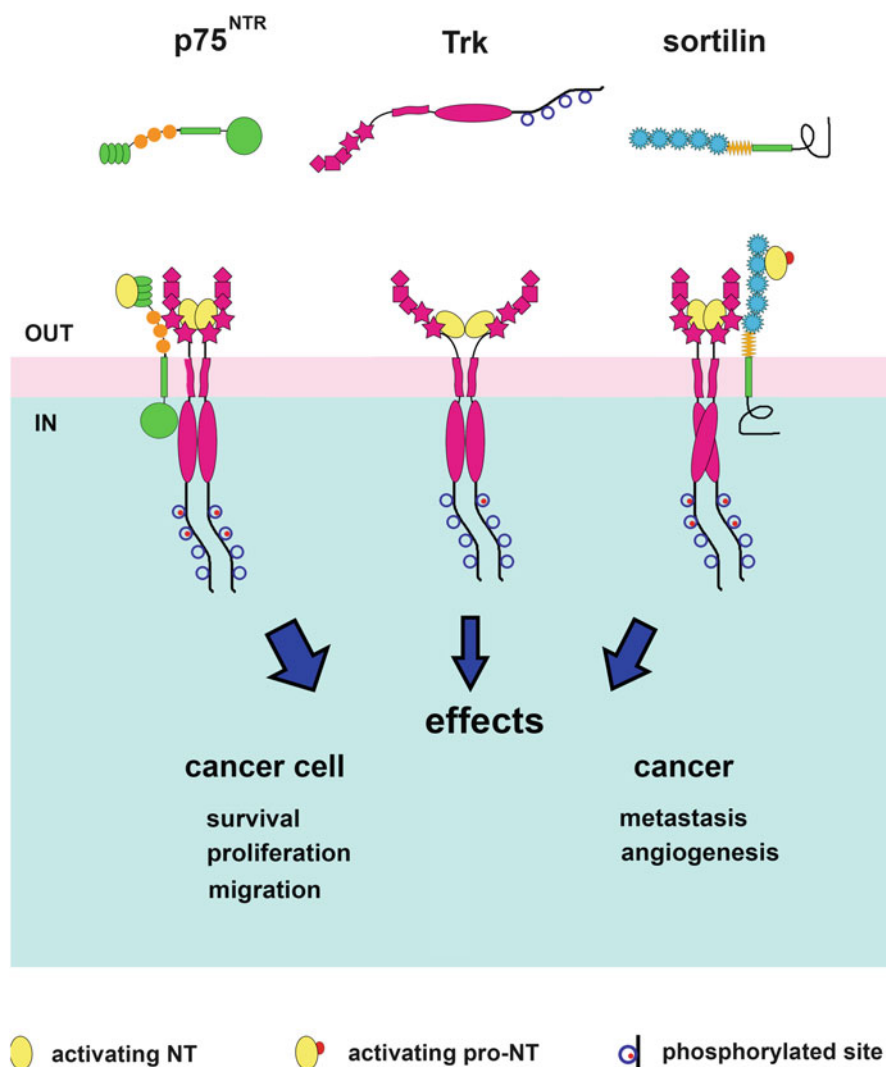
### 2.2.1 Effects of Trk Activation by NT Binding

Classical stimulatory effects are induced in cancer cells upon the binding of their TrkA or TrkB by their specific NTs, NGF, and BDNF (Figs. 1 and 2). Most frequently, stimulations are induced by activation of TrkB, expressed at high level in many cancers such as neuroblastomas, breast cancers, large cell neuroendocrine carcinomas, lung non-small carcinomas and adenocarcinomas, B-cell lymphoblastic malignancies including acute leukemias and myelomas (Croucher et al. 2015; Kim et al. 2016; Sinkevicius et al. 2014). Interestingly, glioblastoma cells inactivated by down-regulation of their TrkB, are re-activated of their aggressiveness by the fusion of exosomes rich in TrkB, released by adjacent cells (Pinet et al. 2016). Stimulatory effects similar to those of TrkB have been observed in cancer cells rich of TrkA (Vera et al. 2014), especially when associated with sortilin (Fig. 1), a multifunctional, non-G protein-coupled receptor, aggressive to breast cancer cells even when signaling alone (Roselli et al. 2015). The responses to Trk receptor activation depend on many processes activated in the course of the signaling pathways, including the stimulation of gene expression, triggered by ERKs and AKT at different targets. The final responses in cancer cells include processes such as proliferation, aggressiveness, and metastases (Fig. 2).

The oncogenic properties induced by NT binding, discussed so far, concern especially TrkA and TrkB. TrkC, which is partially homologous to TrkA and TrkB, is known to play a suppressor role in both neural and non-neural cancer cells, which is independent on its activation by NT3 (Genevois et al. 2013; Zhang et al. 2014). Mechanistically, this effect depends on a property of the receptor, the activation of cancer cell apoptosis, which is associated with good prognosis for the patients (Genevois et al. 2013; Zhang et al. 2014). Interestingly, the protective effect of TrkC is withdrawn by NT3. Binding of the latter, in fact, stimulates cancer cell proliferation with metastases in the brain (Louie et al. 2013; Lawn et al. 2015).

### 2.2.2 Activation of Trks by Fusion of Their Genes

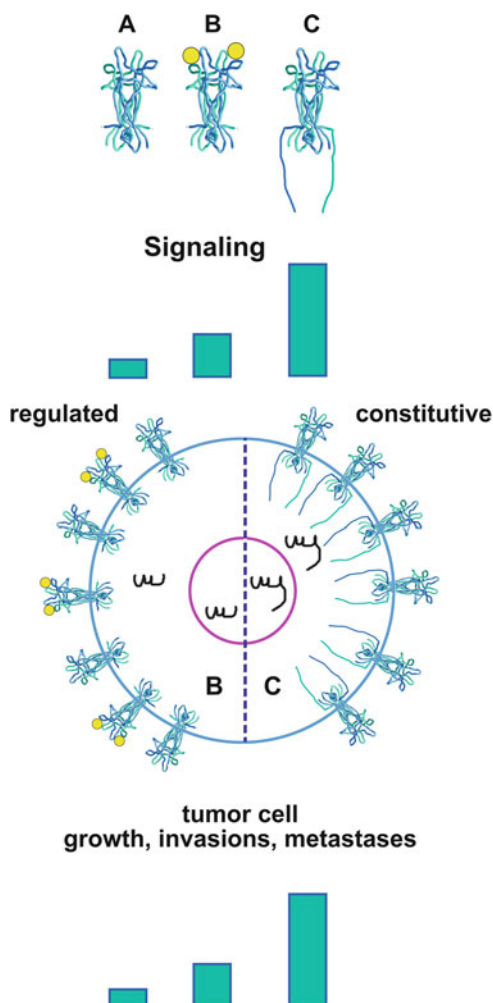
Responses of Trks induced independently on their NT binding were first reported several years ago. Initially these responses were attributed to the proto-oncogene role of NTRK1 and NTRK2, the genes encoding TrkA and TrkB. Mechanistically, however, this proto-oncogene role remained undefined for quite some time.



**Fig. 1** Signaling and effects of the Trk A and TrkB, operative upon dimerization (*center*) and complexed with p75<sup>NTR</sup> (*left*) or sortilin (*right*). NTs and proNTs (yellow) are bound to the two types of receptors, a condition that activates receptor signaling. The different thickness of the arrows indicates that the signaling of the Trk receptor and its complexes differ in intensity. The processes listed below refer to the effects triggered by receptor activations in cancer cells (*left*) and also in multicellular complexes and in in vivo cancers (*right*). Modified from Fig. 1 of Meldolesi (2017)

Growing studies, carried out during the last two decades, have revealed the importance of NTRK1 and NTRK2 fusions with other, distinct genes such as those encoding tropomyosin-3, the lamin A/C and a few others. In terms of frequency, these fusions are quite variable among cancers (from <1 to ~3% in common cancer

**Fig. 2** Comparison of the two types of Trk activation: by receptor activation (*B*) and by NTRK gene fusion (*C*). The top list shows the structure of Trk homotypic dimers (modified from the Fig. 1 of McDonald and Chao 1995), resting (*A*), activated by NTs (*B*) and encoded by a fusion of NTRK (from the 3') to another gene (from the 5'). The data below illustrate the signaling entity of *A*, *B*, and *C*. The cell drawn in the *middle* is shown composed by two halves, *B* and *C* (*left* and *right middles*, respectively). Notice the two, differentially structured genes in the nucleus, their transcripts in the cytoplasm, the different structure and density of the receptors at the cell surface. The *bottom* data shows the responses, including increases of cell growth, aggressiveness, and metastases, induced by Trk in *A*, *B*, and *C* cells



types, such as the lung and colon-rectum cancers; approaching 100% in rare types of tumor, such as mammary secretory carcinoma and congenital fibrosarcoma) (Ricciuti et al. 2017; Vaishnavi et al. 2015). In terms of effects these fusions were found to increase dramatically the oncogenic potential of Trks (Ardini et al. 2014; Doebele et al. 2015; Vaishnavi et al. 2013). Mechanistically, NTRK fusions result in the stimulated gene expression, increased surface density of the encoded receptors, and constitutive operation of their signalling (Fig. 2).

Over a decade ago, oncogenic fusions (to the TEL gene ETV6) have been reported also for NTRK3, the gene encoding TrkC (Bourgeois et al. 2000). Also these fusions were found to result in gene-induced responses in a few types of cancers such as the infantile fibrosarcoma. In addition, the various fused NTRKs were shown to undergo point mutations, with ensuing further increase of the cancer

outgrowth potential of their effects (Khotskaya et al. 2017). Gene fusions and mutations are now considered markers of various types of cancer, to be analyzed before specific decisions in therapy are made.

### 3 Anti-Cancer Drugs

The growing role of drugs in cancer cells has increased considerably the interest about NT receptors. In various patients these receptors have been useful to identify specific biomarkers, critical for diagnosis, therapy, and provision of clinical outcomes (Hondemarck 2012). More important, however, has been the development, for various types of cancer, of NT receptor inhibitors illustrated from here on.

#### 3.1 *Anti-Trk Drugs*

The interest in new drugs started to grow upon the identification of Trk receptors as stimulators of several cancers. Protection induced in both cell lines and living mice by TrkA and TrkB inhibitors had been reported some years ago. Specifically, proliferation and aggressiveness of pancreatic, neuroblastoma and neuroendocrine tumors were attenuated by induction of cell apoptosis, with ensuing animal survival (Demir et al. 2016; Hondemarck 2012; Odate et al. 2013). Considerable improvements occurred recently upon the introduction in research of a second generation of anti-Trk drugs, at present under analysis for medical employment. Entrectinib, an exquisitely potent pan-Trk inhibitor active also on ROS1 and ALK oncoproteins, emerged as a promising agent targeted against various cancers: non-small cell lung cancers, glioblastomas, colorectal, renal, and mammary carcinomas, and melanomas (Ardini et al. 2016; Drilon et al. 2017; Iyer et al. 2016; Rolfo et al. 2015; Russo et al. 2016). Additional drugs, such as GTX-186, induce both anti-cancer and anti-inflammatory actions (Narayanan et al. 2013). Another drug, LOXO-101, specific for Trk receptors, was found to inhibit several cancers (Doebele et al. 2015). Interestingly, both entrectinib and LOXO-101 are active also on patients with tumors harboring NTRK gene fusions (Doebele et al. 2015; Drilon et al. 2017). At present the potential of the latter anti-Trk drugs in human therapy is under control in phase 2 trials (Ardini et al. 2016; Doebele et al. 2015; Russo et al. 2016). Phase 1 studies of other Trk inhibitors, including GNF-5837, MGCD516, PLX7486, DS7486, DS6051b and TSR-011, are underway (Doebele et al. 2015).

Additionally, recently developed Trk inhibitors are under investigation by a few industries. Among them are GNF-4256 and GNF-5837 (Albaugh et al. 2012; Doebele et al. 2015), HS-345 (Seo et al. 2013), AZ623 (Zage et al. 2011), AZD6918, and CEP701 (Tajbakhsh et al. 2017). Other structurally distinct pan-Trk inhibitors, WO2015042088 A1 (Bernard-Gauthier and Schirrmacher 2016), imidazopyridazine (Choi et al. 2015) and (Z)-3-((methoxy-1H-indol-3-yl)

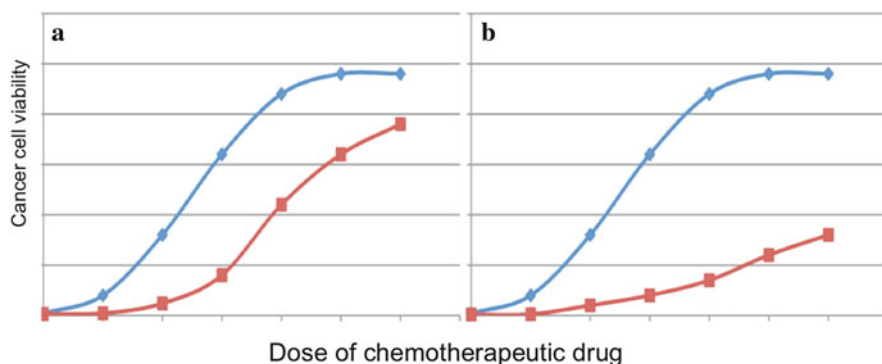
methylene)-2-oxindole (Tammiku-Taul et al. 2016), are of interest because of their therapeutic potential. Inhibition of Trks is not unique to small drug molecules but is induced also by monoclonal antibodies (Demir et al. 2016) and specific siRNAs (Bernard-Gauthier and Schirmacher 2016). In addition, a role is now recognized to inhibitors of  $p75^{\text{NTR}}$ . In kidney, breast, and other cancer cells,  $p75^{\text{NTR}}$  induces effects similar to those of TrkA and TrkB, i.e. stimulations (Alshehri et al. 2017; De la Cruz-Morcillo et al. 2016; Saltari et al. 2016; Zhou et al. 2016; Zhu et al. 2013), that are clocked-down by anti- $p75^{\text{NTR}}$  drugs, monoclonal antibodies, and siRNAs (Demir et al. 2016; Zhang et al. 2015). In the cells of the above cancers, therefore, stimulatory responses are induced by both Trk and  $p75^{\text{NTR}}$  receptors.

The evidence we have summarized documents the present interest for inhibitors and other tools that inhibit signaling of Trks and also of  $p75^{\text{NTR}}$ , effective in the therapy of many tumors. The developments expected for the next several years, in addition to the discovery of new drugs, will lead to the detailed characterization of drugs already available, necessary for their choice and employment in human therapy.

### 3.2 *Combination of Anti-Trk and Chemotherapeutic Drugs*

The mechanistic properties of the anti-Trk agents, based on the inhibition of receptor function, expression, binding, signaling, and metabolism (Demir et al. 2016), are profoundly different from the properties of chemotherapeutic, anti-cancer drugs. Because of these differences, the combination of agents of the two types is not expected to worsen their side effects. Rather, combined administrations often induce highly synergistic results, with elimination of some resistances (Fig. 3) and without serious disadvantages. Here I will summarize some results obtained by the combination of anti-Trk and anti-cancer drugs.

Among the available anti-Trk drugs, the relatively old k252a, analogously to other agents such as GW-441775 and ANA-12, synergizes various anti-cancer drugs to arrest the growth of large B-cell lymphomas and Ewing sarcomas (Dubanet et al. 2015; Heinen et al. 2016). Similar results have been obtained in neuroblastomas (Croucher et al. 2015; Iyer et al. 2010; Li et al. 2015), gliomas, glioblastomas (Pinet et al. 2016; Wang et al. 2015) and breast (Chakravarthy et al. 2016; Tajbakhsh et al. 2017) cancers by recently developed anti-Trk drugs combined to classical anti-cancer drugs. Among the anti-Trk drugs already employed are entrectinib, LOXO-101, GNF-4256, AZ623, and AZD6918 (Croucher et al. 2015; Heinen et al. 2016; Tajbakhsh et al. 2017; Zage et al. 2011). The anti-cancer chemotherapeutic drugs already employed in combination with anti-Trk drugs include irinotecan, paclitaxel, topotecan, lestaurtinib, doxorubicin, cisplatin, and etoposide, that affect a variety of nuclear and cytoplasmic structures and functions (Croucher et al. 2015; Iyer et al. 2010; Zage et al. 2011; Tajbakhsh et al. 2017). Similar results were obtained using, as anti-cancer agent, the monoclonal antibody rituximab, generated against B cells from lymphoproliferative diseases (Dubanet et al. 2015).



**Fig. 3** Comparative effects of a chemotherapeutic drug administered alone (*red*) or in combination with an anti-Trk drug (*blue*). (a) Illustrates the higher potency of the combined drugs with respect to the anti-cancer chemotherapeutic drug alone. (b) Shows that the resistance to the chemotherapeutic drug, established by cancer cells during their treatment, is greatly attenuated by the combined treatment of the chemotherapeutic drug together with an appropriate anti-Trk drug

## 4 Conclusions

Recent studies have confirmed and expanded knowledge of TrkA and TrkB, two receptors highly expressed in many cancers and known to stimulate their growth. At least some established properties of these receptors have been interpreted in mechanistic terms. For example, the proto-oncogenic role of TrkA and TrkB is now known to depend on fusions and mutations of their encoding genes, observed with greatly different frequency in various cancers; TrkC function is variable depending on its NT3 binding, a process that converts the receptor from protector of the cell to promoter of cancer.

Activation by NT binding of TrkA, and even more of TrkB, are the critical processes, among the most relevant in oncology. Knowledge growth about these processes is therefore of great relevance. The most important progress in the field has been in pharmacology. Successful investigations, carried out primarily on cell lines and living mice, are based on the employment of a second generation of anti-Trk drugs that affect the main properties of cancers, including cell proliferation, invasion, and metastasis. Also interesting is the action of some such drugs against inflammation by mechanisms different from those of classical anti-inflammatory drugs (Narayanan et al. 2013). At present, some of the anti-Trk drugs are employed for trials, the task being their introduction in human therapy.

An important development of cancer therapy has started, based on the combined treatment with anti-Trk and classical anti-cancer chemotherapeutic drugs. This approach, already studied in several cancers, has revealed protection of cell lines, xenograft tumors, and in vivo mouse tumors (Chakravarthy et al. 2016; Croucher et al. 2015; Dubanet et al. 2015; Iyer et al. 2010; Lawn et al. 2015; Li et al. 2015; Tajbakhsh et al. 2017; Zage et al. 2011). By the use of the combinations, significant

advantages have been obtained including (1) the role of the Trk receptors in the pathogenesis of various cancers, important for the choice of their therapy; (2) the experience about anti-Trk drugs, active by mechanisms different from chemotherapeutic drugs. The differential properties of the two types of drugs permit the doses of the chemotherapeutic drugs to be reduced when employed in combination with anti-Trk drugs (Fig. 3a). (3) the attenuation of cancer resistances to single drugs (Fig. 3b), with no increase of side effects. Based on these findings, the already considerable interest for anti-Trk drugs in cancer therapy will further increase during the next few years.

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# Gaseous Signaling Molecules in Cardiovascular Function: From Mechanisms to Clinical Translation



Sung Ryul Lee, Bernd Nilius, and Jin Han

**Abstract** Carbon monoxide (CO), hydrogen sulfide (H<sub>2</sub>S), and nitric oxide (NO) constitute endogenous gaseous molecules produced by specific enzymes. These gases are chemically simple, but exert multiple effects and act through shared molecular targets to control both physiology and pathophysiology in the cardiovascular system (CVS). The gases act via direct and/or indirect interactions with each other in proteins such as heme-containing enzymes, the mitochondrial respiratory complex, and ion channels, among others. Studies of the major impacts of CO, H<sub>2</sub>S, and NO on the CVS have revealed their involvement in controlling blood pressure and in reducing cardiac reperfusion injuries, although their functional roles are not limited to these conditions. In this review, the basic aspects of CO, H<sub>2</sub>S, and NO, including their production and effects on enzymes, mitochondrial respiration and biogenesis, and ion channels are briefly addressed to provide insight into their biology with respect to the CVS. Finally, potential therapeutic applications of CO, H<sub>2</sub>S, and NO with the CVS are addressed, based on the use of exogenous donors and different types of delivery systems.

**Keywords** Carbon monoxide • Cardiovascular • Hydrogen sulfide • Ion channel • Mitochondria • Nitric oxide • Translational medicine

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## 1 Introduction

The gases carbon monoxide (CO), hydrogen sulfide (H<sub>2</sub>S), and nitric oxide (NO) have been viewed as toxic ligands because, in biological systems, they can bind to hemoglobin (Hb) or mitochondrial cytochrome c oxidase, thereby blocking oxygen (O<sub>2</sub>) transport to tissues or inhibiting energy production, respectively (Kajimura et al. 2010). The heart and brain are most vulnerable to CO, H<sub>2</sub>S, or NO-induced acute hypoxia, which can be attributed to their high oxygen demands. However, beyond their toxicities, the diverse physiological actions of these biologic

gases have attracted much interest as new medicinal targets and physiological regulators that convey signal transduction via membrane- or receptor-independent signaling (Semenza and Prabhakar 2012). Therefore, an innovative reconsideration of the roles of these gasotransmitters may open new opportunities to intervene in numerous diseases, including cardiovascular complications. CO, H<sub>2</sub>S, and NO are synthesized naturally in the body (Lang et al. 2007; Basudhar et al. 2016; Wang 2014; Cebova et al. 2016; Lloyd 2006) and their absence causes pathophysiological conditions such as hypertension (Kajimura et al. 2010; Cebova et al. 2016). Their discovery and characterization of their mechanisms of action have altered the conventional paradigm of intercellular signaling (Kajimura et al. 2010; Mistry and Brewer 2017). CO, H<sub>2</sub>S, and NO are considered gasotransmitters; i.e., small gaseous signaling molecules with a simple molecular composition and structure (Table 1), with the capability of traveling rapidly both intracellularly and intercellularly (Thomas et al. 2015). Although CO, H<sub>2</sub>S, and NO have simple chemical structures (Fig. 1 and Table 1), the regulation of their production and their biological effects is complex because of the multiple interactions that occur among these gasotransmitters based on their individual concentrations, spatial localization, and temporal regulation (Kajimura et al. 2010). H<sub>2</sub>S can be ionized and its solubility is driven by pH, and perhaps its various forms should not be considered gases under these circumstances (e.g., hydrosulfide anion [HS<sup>-</sup>]; sulfide anion [S<sup>2-</sup>]). The solubilities of NO and CO, like any other gases, are governed by several parameters, such as the temperature, molarity of the solution, and partial pressure of the gas in the gas phase. In contrast to H<sub>2</sub>S, the solubilities of NO and CO are pH-independent. Other gaseous molecules including ammonia (NH<sub>3</sub>), carbon dioxide (CO<sub>2</sub>), hydrogen gas (H<sub>2</sub>), methane (CH<sub>4</sub>) have been suggested as potential gasotransmitters (Wang 2014; Meigh et al. 2013; Wang et al. 2010; Takahashi et al. 2012); however, detailed descriptions of these gases are beyond the scope of our review.

Whereas the endogenous levels of CO, H<sub>2</sub>S, and NO in the circulation or in tissues are relatively low (Table 1), the molecular and cellular effects of these molecules are profound and extremely widespread from bacteria to plants and mammalian cells (Kajimura et al. 2010; Wang 2014). In addition to their involvement in O<sub>2</sub> sensing (Semenza and Prabhakar 2012), these endogenously produced gases work as neurotransmitters and signal mediators to target enzymes, ion channels (Fig. 2 and Table 2), mitochondrial respiration control (Fig. 3), and different transporters, and can either antagonize or facilitate each other's cellular effects (Semenza and Prabhakar 2012; Cebova et al. 2016; Takahashi et al. 2012). Broadly speaking, CO, H<sub>2</sub>S, and NO can regulate vasodilation, directly inhibit apoptosis, increase the expression of anti-apoptosis genes, and activate antioxidants, while inhibiting inflammatory actions and inducing autophagy in the body (Cebova et al. 2016; Szabo 2010). As reported previously, dietary supplementation with inorganic nitrates affords a tremendous opportunity for developing a simple, cheap, and effective NO donor for treating cardiovascular disease (CVD) (Papapetropoulos et al. 2015a). Notably, the mechanisms of well-established drugs have now been shown to involve gasotransmitters (Szabo 2010; Jacobson 2009). For example, the therapeutic effect of cholesterol-lowering statin drugs can be ascribed to the

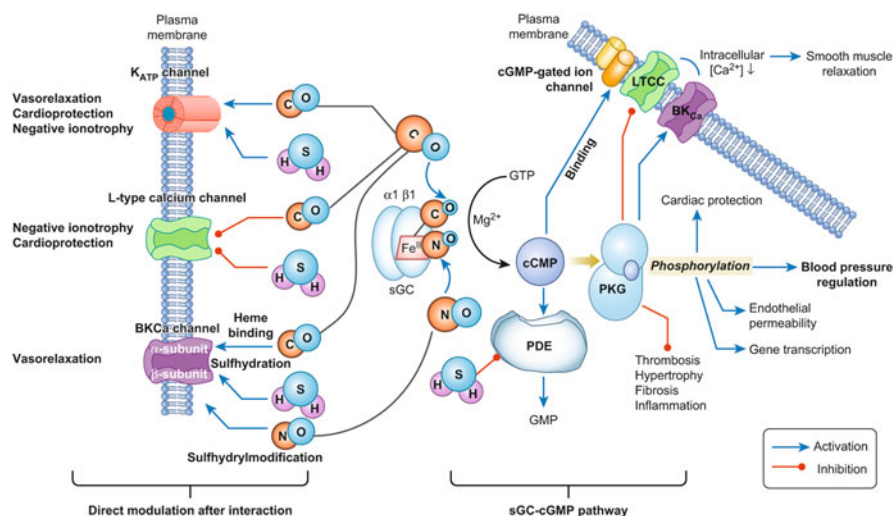
**Table 1** Physiochemical properties of CO, H<sub>2</sub>S, and NO, as well as enzymes involved in their production

Property	Carbon monoxide (CO)	Nitric oxide (NO)	Hydrogen sulfide (H <sub>2</sub> S)
Molar mass (g/mol)	28.01	30.01	34.08
Dipole moment (D, debye)	0.11	0.16	0.97
Solubility (g/100 g H <sub>2</sub> )/ 20°C	0.0028	0.0062	0.4
Affinity for metal ions	++	+++	Not applicable
Ionized form in water	Neutral CO	NO <sup>+</sup> (nitrosonium ion)	Hydrosulfide anion (HS <sup>-</sup> )
		NO <sup>-</sup> (nitroxide ion)	Sulfide anion (S <sup>2-</sup> )
			HS <sup>2-</sup>
			Thiosulfate (S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> )
			Sulfate (SO <sub>4</sub> <sup>-</sup> )
Possible chemical reaction	Inert	Reducible or oxidizable	Reducible
Bound form of hemoglobin	Carboxyhemoglobin	S-nitrosohemoglobin (SNO-Hb)	Sulfhemoglobin
Synthetic enzymes	HO-1 (caveolae, mitochondria, nucleus)	Endothelial NOS (caveolae) Neuronal NOS	CBS (cytoplasm, nucleus, mitochondria)
	HO-2	Inducible NOS	CSE (cytoplasm, nucleus, mitochondria)
			MST (mitochondria)
Blood concentration	nM–μM	Low nM	High nM–low μM
Half-life (in vivo)	Minutes	Seconds	Seconds to minutes
Activation signal in the vasculature	Acetylcholine	Acetylcholine	Acetylcholine
Phenotype related with gene knockout	Sensitive to I/R injury	Hypertension	Hypertension
Chemical modification of protein	Carbonylation at proline, threonine, lysine, and/or arginine residue (Cattaruzza and Hecker 2008)	S-nitrosylation at cysteine residues	S-sulphydration at cysteine residues

*CAT* cysteine aminotransferase, *CBS* cystathionine-β-synthase, *CSE* cystathionine-γ-lyase, *HO* heme oxygenase, *I/R* ischemia/reperfusion injury, *MST* 3-mercaptopyruvate sulfurtransferase, *NOS* nitric oxide synthase







**Fig. 2** Convergence of CO, H<sub>2</sub>S, and NO activities in the cardiovascular system via either sGC–cGMP-dependent or -independent pathways. Both CO and NO activate sGC by interaction with the heme moiety of sGC, and subsequent cGMP production is accelerated. Although H<sub>2</sub>S does not bind to the heme moiety of sGC, H<sub>2</sub>S enhances the bioavailability of cGMP through PDE inhibition. Multiple intracellular proteins such as cGMP-gated ion channels, PKG, and allosteric sites and catalytic sites on certain PDEs are under the control of cGMP. Activation of PKG signaling by cGMP binding can induce protein phosphorylation and thus affect the activity of ion channels, such as LTCC and BK<sub>Ca</sub>, which are involved in smooth muscle relaxation. In contrast to the sGC–cGMP-mediated signaling pathway, all three gases can activate K<sub>ATP</sub>, LTCC, and BK<sub>Ca</sub> ion channels through a specific protein modification, thus leading to vasorelaxation and/or cardioprotection against reperfusion injury in the heart. BK<sub>Ca</sub> big-conductance Ca<sup>2+</sup>-activated potassium channel, cGMP cyclic guanosine monophosphate, K<sub>ATP</sub> channel, ATP-sensitive potassium channel, LTCC L-type calcium channel, PDE phosphodiesterase, PKG protein kinase G, sGC soluble guanylyl cyclase

activation and increased expression of endothelial NO Synthase (eNOS) through the involvement of multifaceted signaling mechanisms, thereby improving cardiovascular function, which could be implicated in statin-associated cardiovascular protection (reviewed in Balakumar et al. 2012). In addition, the PDE 5 inhibitors tadalafil (Salloum et al. 2009) and cinaciguat (BAY 58-2667; a novel NO-independent activator of soluble guanylate cyclase (sGC)) may further confer a cardioprotective role that is involved in cGMP-activated G protein kinase G (PKG)-dependent H<sub>2</sub>S generation (Salloum et al. 2012). However, a greater understanding is required to develop effective therapeutics and translate these into clinical settings (Szabo 2010; Polhemus and Lefer 2014). A better understanding of how these molecules are related with each other will help in developing more efficient therapeutic strategies to maximize the benefits of both signaling molecules (Polhemus and Lefer 2014). In this review, the regulatory roles of CO, H<sub>2</sub>S, and NO are briefly summarized regarding their effects on ion channels (Fig. 2 and Table 2) and mitochondria (Fig. 3) in the cardiovascular system. These three gases

**Table 2** Direct ion channel-modulatory effects of CO, H<sub>2</sub>S, and NO in the cardiovascular system

Ion channel	Cell or tissue	Gasotransmitter	Mode	Functional consequence
<i>Potassium (K<sup>+</sup> channel)</i>				
Ca <sup>2+</sup> -activated K channel (K <sub>Ca</sub> )	VSMC	CO	Activation through CO binding	Vasorelaxation independent of cGMP (Wang et al. 1997)
Slowly activating delayed rectifier K <sup>+</sup> channel	Ventricular myocytes (guinea pig)	H <sub>2</sub> S (Na <sub>2</sub> S) NO	Activation resulting in APD shortening and suppression of Ca <sup>2+</sup> overload	Cardioprotection (Bai et al. 2005)
ATP-sensitive K channel (K <sub>ATP</sub> channel)	VSMC	H <sub>2</sub> S (gas or NaHS)	Activation by sulfhydrylation (Mustafa et al. 2011)	Vasorelaxation (Soni et al. 2010)
		CO (CORM-2)		Cardioprotection during I/R (Tang et al. 2005; Jaggar et al. 2005)
	Cardiomyocyte	H <sub>2</sub> S	Activation	Negative inotropic effect (Geng et al. 2004a)
	Sinoatrial node pacemaker cell	H <sub>2</sub> S	Activation	Negative chronotropic effect
Big-conductance Ca <sup>2+</sup> activated K <sup>+</sup> channel (BK <sub>Ca</sub> )	Endothelial cell	CO	Activation by interaction with heme of BK <sub>Ca</sub> ? or interaction with cyanide-sensitive site in BK <sub>Ca</sub>	Vasorelaxation (Jaggar et al. 2005; Dong et al. 2007; Wang et al. 1997)
	Rat tail artery VSMC	NO	Modification of the sulfhydryl group of the K <sub>Ca</sub> channel (Wu et al. 2002)	Vasorelaxation
	Rat tail artery VSMC	CO	Activation by heme binding (Jaggar et al. 2005; Wu et al. 2002)	Vasorelaxation
Voltage-operated Ca <sup>2+</sup> channel (VOCC)	Rat cardiomyoblast H9c2 cell	H <sub>2</sub> S (NaHS)	Inhibition (Avanzato et al. 2014)	
T-type Ca <sup>2+</sup> channel	Rat cardiomyoblast H9c2 cell	H <sub>2</sub> S (NaHS)	Inhibition	Cardioprotection against I/R (Avanzato et al. 2014)

(continued)

**Table 2** (continued)

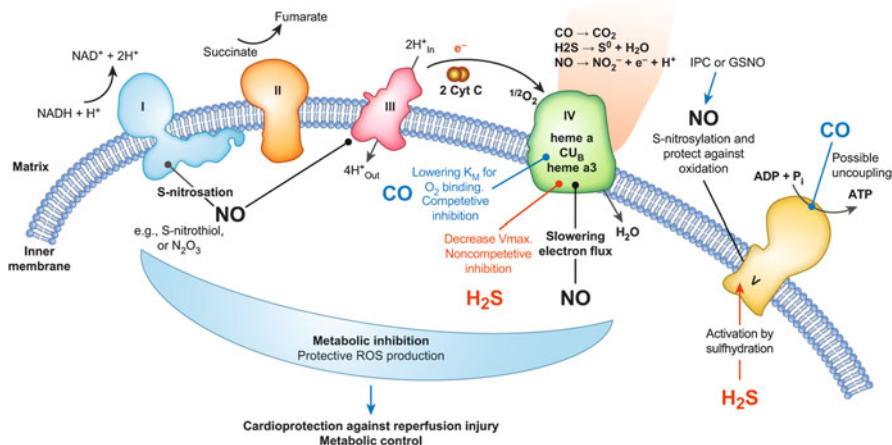
Ion channel	Cell or tissue	Gasotransmitter	Mode	Functional consequence
Ca <sup>2+</sup> -activated small-conductance K channel	Endothelial cell/smooth muscle cell	H <sub>2</sub> S	Activation by sulfhydration	Vasorelaxation independently of NO (Mustafa et al. <a href="#">2011</a> )
Ca <sup>2+</sup> -activated intermediate-conductance K channel	Endothelial cell/smooth muscle cell	H <sub>2</sub> S	Activation by sulfhydration	Vasorelaxation independently of NO (Mustafa et al. <a href="#">2011</a> )
<i>Calcium (Ca<sup>2+</sup>) channel</i>				
L-type Ca <sup>2+</sup> channel (LTCC)	Cardiomyocyte	H <sub>2</sub> S	Inhibition	Reduced cardiac contractility (Sun et al. <a href="#">2008</a> ; Zhang et al. <a href="#">2012</a> )
		CO	Inhibition of $\alpha 1c$ subunit	Cardioprotection against I/R Reduced cardiac contractility (Scragg et al. <a href="#">2008</a> )
	Ferret right ventricular myocyte	NO (RSNO)	Activation by S-nitrosylation of LTCC	Increase in cardiac basal contractility (Hare <a href="#">2003</a> )
	Rat heart	NO derived from eNOS and nNOS	Inhibition of LTCC by S-nitrosylation	Cardioprotection during I/R (Sun et al. <a href="#">2006</a> ) Anti-arrhythmia following I/R (Burger et al. <a href="#">2009</a> )
<i>Sodium channel</i>				
Na <sup>+</sup> channel, Na <sub>v</sub> 1.5	Heart	CO	Activation of NO synthase, leading to NO-mediated nitrosylation	Prolongation of the cardiac action potential and pro-arrhythmic (Dallas et al. <a href="#">2012</a> )
Voltage-gated Na <sup>+</sup> channels	Ventricular myocyte	NOS-mediated NO		Leads to the appearance of a persistent sodium (Na <sup>+</sup> ) current that fails to inactivate completely at depolarized membrane potentials

(continued)

**Table 2** (continued)

Ion channel	Cell or tissue	Gasotransmitter	Mode	Functional consequence
<i>Chloride (Cl<sup>-</sup>) channel</i>				
Cl <sup>-</sup> channel	Cardiomyocyte preparation	H <sub>2</sub> S	Inhibition	Cardioprotection during I/R
	Rat heart lysosomal vesicles	H <sub>2</sub> S	Inhibition (Malekova et al. 2009)	
<i>Others</i>				
TRP ion channels such as TRPC1, TRPC4, TRPC5, TRPV1, TRPV3, TRPV4, and TRPC6	Recombinant protein	NO	Activation via cysteine S-nitrosylation (Kozai et al. 2015; Yoshida et al. 2006)	
	A7r5 vascular myocyte	NO (SNAP)	Suppression via cGMP-PKG-dependent phosphorylation (Takahashi et al. 2008)	
TRPA1	Sensory neuron	HNO (Eberhardt et al. 2014) or polysulfide (Miyamoto et al. 2017) formed from NO and H <sub>2</sub> S	Activation by formation of disulfide bonds	Neuronal stimulation-mediated vasodilation
		Polysulfide		
CX43 hemichannel	<i>Xenopus laevis</i> oocyte	CO (CORM-2)	Inhibition by carbonylation of Cx43 or Cx46 (Leon-Paravic et al. 2014)	Cataract formation (Retamal et al. 2009)
	<i>Xenopus laevis</i> oocytes lens	NO (GSNO)	SNO modification COOH-terminal domain of Cx46 hemichannel	
Ryanodine receptor RyR1	Skeletal muscle	S-nitrosothiol	Hypernitrosylation (Xu et al. 1998)	Sensitize the muscle to Ca <sup>2+</sup> induced Ca <sup>2+</sup> release
	Canine heart		Hypernitrosylation (Xu et al. 1998)	
Cl <sup>-</sup> /HCO <sub>3</sub> <sup>-</sup> exchanger	A7r5 vascular smooth muscle cell	NaHS	Activation	Vasorelaxation via lowering pH (Lee et al. 2007)

I/R ischemia/reperfusion, TRP transient receptor potential, VSMC vascular smooth muscle cell



**Fig. 3** Cardioprotection mediated by CO, H<sub>2</sub>S, and NO via mitochondrial respiratory control. Complex IV contains Fe–Cu clusters and is the major mitochondrial respiration-inhibition site involving CO-, H<sub>2</sub>S-, and NO-binding. Inhibition of complex IV, which occurs upon reperfusion by CO, H<sub>2</sub>S, or NO, leads to the suppression of calcium overload and excessive ROS production, and thereby confers mitochondrial-mediated cardioprotection. Under physiological conditions, H<sub>2</sub>S activates complex V activity through sulphydryl modification

can interact with each other, and crosstalk among them is briefly addressed. Given the great potential for off-target effects, achieving targeted and localized delivery is especially important to harness the potential therapeutic efficacy of these gases (Qian and Matson 2017). Therefore, we have outlined several methods for developing and delivering CO- (Table 3), H<sub>2</sub>S- (Table 4), and NO-donor molecules (Table 5). Finally, we briefly address the clinical translation of CO, H<sub>2</sub>S, and NO for treating CVD.

## 2 Endogenous Synthesis of CO, H<sub>2</sub>S, and NO

It has been suggested that some membranes exhibit extremely low permeability to gases and facilitate movement of gases in ways that are not explained by the solubility–diffusion model (Walter and Gutknecht 1986). For example, gastric glands have no demonstrable permeability to the gases CO<sub>2</sub> and NH<sub>3</sub> (Cooper et al. 2002). Based on its polarity, which is often expressed as the dipole moment, H<sub>2</sub>S has the lowest permeation rate through lipid bilayers (H<sub>2</sub>S << NO < CO) but displays the greatest water solubility (Kajimura et al. 2010). It appears that specific channels including aquaporin 1 (AQP1) (Cooper et al. 2002), an ion exchanger (Jennings 2013), or other less characterized gas channels can augment the movement of some gases across some membranes. The speed of transit of CO, H<sub>2</sub>S, and NO across membranes and their diffusion rates are important parameters in determining their effective concentration at a given target (Kajimura et al. 2010). Although the

**Table 3** CO donors and their clinical implications in the cardiovascular system

CO donor	Mode	Applications
CO gas	Gas inhalation	Chronic obstructive pulmonary disease (100–125 ppm) (Bathoorn et al. 2007)
	No by-products, but hard to control dosage	Lung fibrosis (250 ppm) (Clark et al. 2003)
CO donors	Precise control of dosage	
	Controlled rate of release, but short period	
	Potentially toxic byproduct	
CORM-1 [Mn <sub>2</sub> (CO) <sub>10</sub> ]	Soluble in DMSO	Coronary vasodilation in an endothelium-independent manner (Musameh et al. 2006)
	Stable under aerobic condition	
	UV light excitation-induced CO release	
CORMA-A1(Na <sub>2</sub> H <sub>3</sub> BCO <sub>2</sub> )	Water-soluble organic CO donor	Vasorelaxation (Qian and Matson 2017; Motterlini et al. 2005)
	Stable under aerobic condition	Coronary vasodilation in an endothelium-independent manner (Musameh et al. 2006)
	Slow CO-releasing ( <i>t</i> <sub>1/2</sub> ≈ 21 min)	
CORM-2 ([Ru(CO) <sub>3</sub> Cl <sub>2</sub> ] <sub>2</sub> ) [tricarbonyldichlororuthenium (II)]	Soluble in DMSO	Vasorelaxation
	Stable under aerobic conditions	Inflammation
	Fast CO release ( <i>t</i> <sub>1/2</sub> ≈ 1 min)	Cardioprotection during I/R (Soni et al. 2010)
CORM-3 [tricarbonylchloro (glycinato)ruthenium(II)] [Ru(CO) <sub>3</sub> Cl(glycinate)]	Water soluble	Prevention of cardiac graft rejection in mice and rats (Qian and Matson 2017)
	Stable under aerobic condition	Vasorelaxation (Bilban et al. 2008)
	Fast CO release ( <i>t</i> <sub>1/2</sub> ≈ 1 min)	Cardioprotection during I/R (Andreadou et al. 2015; Clark et al. 2003)
		Positive inotropic effect through sGC and Na <sup>+</sup> /H <sup>+</sup> exchanger (Musameh et al. 2006)
		Chronic obstructive pulmonary disease (Qian and Matson 2017)
		Increase of mitochondrial biogenesis in the heart of peritonitis-induced sepsis (Lancel et al. 2009)

(continued)

**Table 3** (continued)

CO donor	Mode	Applications
CORM-401 [Mn(CO) <sub>4</sub> {S <sub>2</sub> CNMe(CH <sub>2</sub> CO <sub>2</sub> H)}]	Manganese as a metal center and oxidant-sensitive CO-release	Vasodilation and angiogenesis (Fayad-Kobeissi et al. 2016) Uncoupling of mitochondrial respiration and inhibition of glycolysis (Kaczara et al. 2015)
<i>Pro-drugs</i>	Decomposition and release at specific sites in the body via enzymatic reaction	
Methylene chloride	Conversion into CO by carbonic anhydrase	Reduces chronic graft deterioration (Martins et al. 2005, 2006)

identification of specific gas channels for CO, H<sub>2</sub>S, and NO will strengthen our understanding of the biology of these gases and their controlled bioavailabilities in the cardiovascular system, the mechanisms whereby different gases permeate membranes are still under investigation. In this section, the characteristics and endogenous production of CO, H<sub>2</sub>S, and NO in the cardiovascular system are described. All three gaseous molecules are produced by specific enzymes, although non-enzymatic production of these gasotransmitters also occurs.

## 2.1 Carbon Monoxide (CO)

CO is a diatomic oxide of carbon with a triple bond that does not dissociate chemically in aqueous solution, owing to its extremely low solubility (354 ml/dl; 44.3 ppm by mass) at standard temperature and pressure (reviewed in refs. (Kajimura et al. 2010; Wu and Wang 2005)). When CO is coordinated with other molecules such as transition metals, its reactivity is increased relative to that of the free form of CO. Compared to NO or H<sub>2</sub>S, both of which possess a free electron, CO is the most biologically stable gasotransmitter owing to its weak chemical reactivity (Table 1). Exogenous CO, an omnipresent air pollutant, is a by-product from the combustion or oxidation of organic matter, coke, and tobacco (Kim et al. 2006).

The rate of CO–Hb saturation from CO inhalation is slow in humans (taking 8–24 h), but is relatively fast in small species such as rats (taking 1–2 h) (Montgomery and Rubin 1971). Once it enters the body, CO is carried by red blood cells, where it binds to the ferrous (Fe<sup>2+</sup>) heme form of Hb, resulting in the production of carboxy-Hb. CO is subsequently eliminated through the lungs, after being displaced by O<sub>2</sub> as the red blood cell traverses along a capillary in the alveolar membrane (Kajimura et al. 2010). Thus, CO, with a half-life of 3–7 h in the human body, might be capable of exerting its effects over longer time periods (Kajimura et al. 2010; Motterlini and Otterbein 2010), and over longer distances compared to NO or H<sub>2</sub>S (Cebova et al. 2016). Heme oxygenase (HO) is a microsomal enzyme that contains a unique heme serving both as a substrate and a catalytic center (Maines 1988). In

**Table 4** H<sub>2</sub>S donors and their clinical implications in the cardiovascular system

H <sub>2</sub> S donor	Mode	Applications
<i>H<sub>2</sub>S gas</i>	Gas inhalation	Hemorrhage (Morrison et al. <a href="#">2008</a> )
	No by-products but hard to control dosage	Suspended animation in mice (Volpato et al. <a href="#">2008b</a> )
<i>Simple H<sub>2</sub>S-donor</i>	Precise control of dosage	
	Controlled rate of release but short period	
	Potentially toxic by-products	
Sodium thiosulfate (Na <sub>2</sub> O <sub>3</sub> S <sub>2</sub> )	Hydrophilic	Prevention of angiotensin II-mediated hypertension (Snijder et al. <a href="#">2015</a> )
Sodium hydrosulfide (NaHS)	Hydrophilic	Cardioprotection during I/R (Chen et al. <a href="#">2016</a> ; Hua et al. <a href="#">2013</a> ; Li et al. <a href="#">2015</a> )
		Vasorelaxation (Snijder et al. <a href="#">2015</a> )
Sodium sulfide (Na <sub>2</sub> S)	Hydrophilic	Vasorelaxation (Cacanyiova et al. <a href="#">2016b</a> ; Tomasova et al. <a href="#">2016</a> )
	Increase in free H <sub>2</sub> S for ~520 min in vivo; ~5 min in vitro	Less cardiac hypertrophy and left ventricular dilation (Nicholson et al. <a href="#">2013</a> )
		Cardioprotection during I/R (Elrod et al. <a href="#">2007</a> )
GYY4137 [morpholin-4-ium 4 methoxyphenyl (morpholino) phosphinodithioate]	Hydrolysis-triggered H <sub>2</sub> S release	Vasorelaxation (Kashfi and Olson <a href="#">2013</a> )
	Slow-release and longer lasting (3–4 h)	Inhibits oxidative stress-induced mitochondrial and cellular injury (Gao et al. <a href="#">2015</a> ; Zhao et al. <a href="#">2014</a> )
		Atherosclerosis (Whiteman et al. <a href="#">2015</a> )
AP67/AP72, AP39	Slow-releasing H <sub>2</sub> S donor (3–4 h).	
	Mitochondria-targeted H <sub>2</sub> S	Prevents glucose oxidase-induced mitochondrial oxidative stress
		Cardioprotection during I/R via inhibition of the mitochondrial permeability transition pore (Chatzianastasiou et al. <a href="#">2016</a> )
SG-1002	Orally active	Pressure overload-induced heart failure (Kondo et al. <a href="#">2013</a> ; Polhemus et al. <a href="#">2015</a> )
Diallyl disulfide	Organosulfur compound	Cardioprotection during I/R in diabetic condition (Yu et al. <a href="#">2017b</a> )

(continued)



**Table 4** (continued)

H <sub>2</sub> S donor	Mode	Applications
Diallyl trisulfide	Insoluble in water	Angiogenesis (Hayashida et al. <a href="#">2017</a> )
<i>Enzymatic regulator</i>		
S-propyl-cysteine	<i>Synthetic cysteine derivatives</i>	Cardioprotection during I/R (Beltowski <a href="#">2015</a> )
S-allyl-cysteine	Substrates for CBS and CSE	
S-propargyl-cysteine (ZYZ-802)		
<i>Hybrid with known drugs</i>	Overcome or reduce drug toxicity with an additional H <sub>2</sub> S-dependent biological activity	
S-aspirin (ACS14)	Aspirin with H <sub>2</sub> S donor (anethole dithiolethione (ADT) and 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADT-OH))	Vasorelaxation (Rossoni et al. <a href="#">2010</a> )
		Antioxidant activity
		Improved gastrointestinal safety profiles (Beltowski <a href="#">2015</a> )
		Cardioprotection during I/R (Rossoni et al. <a href="#">2010</a> )
Carrier-based H <sub>2</sub> S donor	Light-triggered H <sub>2</sub> S donor (gem-dithiol-based donor, nitrobenzyl thioester donor) (Zhao et al. <a href="#">2014</a> )	Under investigation
	Thiol-triggered H <sub>2</sub> S donor (N-benzoylthiobenzamides, S-aroxythiooximes, and perthiols) (Zhao et al. <a href="#">2014</a> )	Under investigation

mammals, small amounts of CO can be continuously produced enzymatically by HO in the presence of both molecular oxygen and the reducing agent NADPH (Fig. 1). Heme degradation does not occur in the presence of CO. It should be mentioned that, under physiological conditions, little free heme is thought to be available as a substrate for HO; thus, CO production appears to be tonically regulated and acute on–off switch is not required (Griscavage et al. [1994](#)). Among the three isoforms of HO, the inducible HO-1 isoform is structurally similar to the constitutive HO-2 isoform (Munoz-Sanchez and Chanez-Cardenas [2014](#)), but is less efficient at degrading heme (Rochette et al. [2013](#)). HO-1 is primarily localized to the endoplasmic reticulum (Maines [1988](#)), but it can be translocated to other subcellular sites such as the nucleus, cytoplasm, nuclear matrix, mitochondria, or peroxisomes, depending on the cellular conditions (Immenschuh et al. [2003](#)). However, the precise distribution and localization of HO-1 in subcellular sites has not been well established. It has been noted that HO-1 induction during hypoxia or heat shock is repressed in various human cells but is induced in rodent cells (Shibahara et al. [2003](#)). A clear reason explaining the interspecies or intertissue differences in HO-1 induction has not been elucidated. Unlike HO-1, HO-2 contains two copies of the

**Table 5** NO donors and their clinical implications in the cardiovascular system

Type of NO donor	Mode	Therapeutic application
NO gas	Gas inhalation (5–20 ppm)	Pulmonary hypertension (5–20 ppm) (Qian and Matson 2017)
	No byproducts, but hard to control dosage	Thrombosis (8 ppm)
	Possible formation of pulmonary irritant NO	Myocardial I/R injury (80 ppm) (Qian and Matson 2017)
Simple NO donors	Precise control of dosage	
	Controlled rate of release, but short period	
	Potentially toxic byproduct	
Glyceryl trinitrate (GTN, nitroglycerin)	Fast NO release	Acute pain associated with angina
		Vasorelaxant effect on all types of vessels (Wang et al. 2002)
		Acute myocardial infarction (Jugdutt 1994)
		Congestive heart failure (Kovick et al. 1976)
Isosorbide mononitrate (ISMN)	Slower NO release	Chronic angina (Serafim et al. 2012)
LA-419	A thiol-containing analogue of isosorbide mononitrate	Vasorelaxation with an antithrombotic activity (Megson and Leslie 2009)
		Inhibition of left ventricular hypertrophy
Sodium nitroprusside (SNP)	NO–iron complex	Rapid reduction of blood pressure in severe hypertension (Serafim et al. 2012)
	Rapid NO release	
	Abrupt hypertension and cyanide toxicity	
Diazeniumdiolates (NONOates), DEA/NO	PROLI/NO; proline adduct of NONOate diethylenetriamine (DETA/NO)	Vasodilation, platelet aggregation, inhibition of blood coagulation (Serafim et al. 2012)
		Inhibition of proliferation of vascular smooth muscle cells (Serafim et al. 2012)
SNAP (S-nitroso-N-acetyl-penicillamine)	Half-life of 9 h	Vasorelaxation
		Increased angiogenesis (Serafim et al. 2012)
<i>Pro-drug</i>	Decomposition and release at specific sites in the body via enzymatic reaction	
Furoxans (1,2,5-oxadiazole-2-oxides)	Production of NO via chemical reaction	Vasorelaxation (Feelisch et al. 1992)
Hybrid with known drug	Overcome or reduce drug toxicity with an additional NO-dependent biological activity	

(continued)

**Table 5** (continued)

Type of NO donor	Mode	Therapeutic application
S-nitroso-captopril	ACE inhibitor captopril + NO donor	Vasorelaxation with less nitrate tolerance (Matsumoto et al. 1995)
NCX-899	ACE inhibitor enalapril + NO donor	Vasorelaxation and suppression of cardiac remodeling (Martelli et al. 2006)
PETN (molsidomine and pentaerythrityl tetranitrate)	Structurally very similar to nitroglycerin	Vasorelaxation with less nitrate tolerance (Fink and Bassenge 1997)
NCX-4016	NO donor with acetyl salicylic acid	Anti-thrombotic and anti-inflammatory potential (Napoli et al. 2001)
		Protection against ulceration of the gastric mucosa (Wang et al. 2002)
		Cardioprotection during I/R (Rossoni et al. 2000)
NCX-6550	NO-releasing pravastatin	Anti-inflammation with inhibitory activity of HMGCoA reductase
NCX-6553	NO-releasing fluvastatin	inhibitor (Serafim et al. 2012; Napoli et al. 2001)

heme regulatory motif in its C-terminal region, which has been suggested to act as an indirect regulatory site affecting substrate affinity through a thiol–disulfide redox switch (Munoz-Sanchez and Chanez-Cardenas 2014). Although HO-2 is constitutively expressed, its expression can also be upregulated by certain stimuli such as glucocorticoids, estrogen, the NOS inhibitor L-NAME, or the HO inhibitor zinc mesoporphyrin (reviewed in Shibahara et al. 2003). Production of cardiac CO can be stimulated by HO-2 in cardiomyocytes, vascular smooth muscle cells (VSMCs) (Ewing et al. 1994), and neurons (Hassall and Hoyle 1997). It is unclear whether cardiac HO-2 is also influenced by stimuli present in other tissue systems. The expression of HO-3, which was first cloned from a rat brain (McCoubrey et al. 1997), is contentious because attempts to demonstrate mRNA and protein production have failed in rats thus far (Hayashi et al. 2004). The structure of the heme–HO complex has an increased affinity for O<sub>2</sub> and a decreased affinity for CO (Sugishima et al. 2003); therefore, in the presence of local CO, HO can escape from autoinhibition during its enzymatic reaction, although this does not occur in the presence of external CO (Kajimura et al. 2010). In tissues, HO-1 can be found in caveolae (a specialized version of membrane-associated lipid rafts) (Patel and Insel 2009), as well as in the nucleus. Heme-L-arginate and heme-L-lysinate induce HO-1 expression (Johnson et al. 1996). Other stimuli including growth factors; oxidative stress consequent to hydrogen peroxide, disease state, infections (reviewed in Wegiel et al. 2015); hormones; dietary antioxidants; natural products; and physiological changes such as heat shock, osmotic pressure, deprivation of glucose, and acidosis can also induce HO-1 (reviewed in Kim et al. 2006). HO-1 can be upregulated in the heart and blood vessels in response to hemodynamic stress in

rats (Yet et al. 1997), as well as during myocardial adaptation following ischemia/reperfusion (I/R) injury in pigs, implying that HO-1 serves an important role in cardiovascular homeostasis (Sharma et al. 1999; Lee et al. 2006). HO-1 transcriptional regulation can be tuned by repressor proteins (e.g., Keap1 and Bach1) that regulate the binding of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) to DNA sequences in target genes as anti-oxidant response elements (reviewed in Niture et al. 2014). Notably, exogenous CO can serve as an inducer of HO-1 expression via indirect Nrf2 activation and its translocation into the nucleus via the mitogen-activated protein kinase (MAPK) signaling pathway, as shown in hepatocytes (Lee et al. 2006). It is unclear whether the induction of HO-1 by CO also occurs in the cardiovascular system. HO-2 is constitutively expressed in atrial and ventricular cardiac myocytes. As with eNOS, HO-2 is localized at the endothelial layer of blood vessels (Lundberg et al. 2015). Besides cigarette smoking, enhanced CO production results from several different diseases such as cirrhosis, chronic obstructive pulmonary disease, sickle cell disease, upper respiratory tract infections, and spontaneous bacterial peritonitis, among others (Rodgers et al. 1994). As summarized in Owens (2010), some drugs such as nicotinic acid, allyl-containing compounds (acetamides and barbiturates), diphenylhydantoin, progesterone, contraceptives, and statins can increase CO production (Owens 2010). Abnormal CO production occurs during hemolysis and is reflected by an elevated CO-Hb level (Coburn et al. 1966). Other minor sources of CO include the oxidation of organic molecules such as phenols, flavonoids, halomethanes, or lipid peroxidation of membrane lipids (Rodgers et al. 1994).

## 2.2 Hydrogen Sulfide

H<sub>2</sub>S is a readily soluble gas in both water and lipids, and can exist as un-dissociated H<sub>2</sub>S, the hydrosulfide anion (HS<sup>-</sup>), and the sulfide anion (S<sup>2-</sup>) (Kajimura et al. 2010; Lowicka and Beltowski 2007). From a chemical point of view (Table 1), H<sub>2</sub>S is the simplest thiol, and as such is a reductant (Beltowski 2015; Kimura 2014). HS<sup>-</sup> can serve as an electrophile extinguisher, with even trace amounts of this ubiquitous mediator affects the detection and actions of endogenously generated electrophiles in biological systems (Nishida et al. 2012). It is difficult to estimate the H<sub>2</sub>S levels in mammalian physiology and the most accurate approach is still under debate, although it is currently accepted that high nanomolar to low micromolar concentrations might be present under non-pathological conditions (Vicente et al. 2016; Vandiver and Snyder 2012). It is generally believed that the aortic concentration of free H<sub>2</sub>S is 20 to 100 times greater than that in other tissues (Levitt et al. 2011). H<sub>2</sub>S catabolism in vivo is less well understood, and most data have been obtained using exogenous H<sub>2</sub>S sources such as a sodium hydrosulfide (NaHS); thus, although they do not have physiological implications, these studies have provided important toxicological insights (Lowicka and Beltowski 2007). H<sub>2</sub>S can bind to methemoglobin to form sulfhemoglobin, which is thought to be an irreversible process, and is

involved in the potentially lethal process of cyanosis owing to the disruption of normal  $O_2$  loading in the blood (Park and Nagel 1984). However, reversible sulfide binding to Hb may work to either create a reserve pool of  $H_2S$  or act as a quencher against toxic levels of  $H_2S$  in the blood. The exact modes of heme–sulfide interactions and sulfide oxidation under physiological and/or pathophysiological condition constitute an emerging research topic (Kolluru et al. 2016).

$H_2S$  is endogenously produced both via enzyme-mediated and non-enzymatic pathways (for example, reduction of thiol-containing molecules), and is also released from intracellular sulfur stores (sulfane sulfur) through the activities of reducing agents (Toohey 2011; Yuan et al. 2016). Four enzymatic pathways for producing  $H_2S$  have been identified to date (Fig. 1). Two pathways are catalyzed by cystathionine  $\beta$ -synthase (CBS) and cystathionine  $\gamma$ -lyase (CSE), which are pyridoxal 5'-phosphate-dependent cytosolic enzymes in the trans-sulfuration pathway, in which homocysteine is metabolized to cysteine (for a review, see Beltowski 2015). The enzymatic activity of  $Fe^{3+}$ -CBS (oxidized form) is 1.7-fold higher than that of  $Fe^{2+}$ -CBS (reduced form) (Taoka et al. 1998). CO or NO binding to  $Fe^{2+}$ -CBS leads to further inhibition of CBS activity (Taoka and Banerjee 2001) by altering its conformation to a physiologically inactive state (Puranik et al. 2006). When CBS is active, cystathionine is produced and kinetic control favors its subsequent conversion by CSE to cysteine (Kabil et al. 2016). CBS can be inhibited by the binding of CO to its heme factor after acute CO production in response to stress, or by disabling mutations associated with CBS-dependent homocystinuria. Whereas CBS is the main  $H_2S$  synthase in the nervous system (Abe and Kimura 1996; Enokido et al. 2005), CSE functions in the cardiovascular system, especially in myocardial cells (Geng et al. 2004a), VSMCs (Zhao et al. 2001; Hosoki et al. 1997), and endothelial cells (Beltowski and Jamroz-Wisniewska 2014). CBS contains a heme cofactor, which operates as a redox sensor and uses S-adenosylmethionine as an allosteric activator (Mikami et al. 2011). The stimulatory effect of  $Ca^{2+}$ /calmodulin (CaM) on CSE activation, as shown in vascular endothelial cells (Yang et al. 2008) is controversial, as the  $H_2S$ -producing activity of purified rat liver CSE was somewhat suppressed by high  $Ca^{2+}$  concentrations and was also independent of CaM (Mikami et al. 2011, 2013). It should be noted that blood  $H_2S$  levels do not necessarily indicate actual endothelial cell  $H_2S$  bioavailability as the amount of  $H_2S$  produced in non-endothelial cells also contributes to its circulating levels (Wang et al. 2015a).

In terms of subcellular organization, CSE and CBS may be found in the cytoplasm, the nucleus, and mitochondria. However, the presence of CSE in endothelial cells (ECs) is controversial. L-cysteine, or its analogs, can activate CSE and thereby increase  $H_2S$  production (Table 3). However, neither CSE nor CBS is as dramatically inducible in the same manner as are iNOS and HO-1 (Lundberg et al. 2015). The physiological signals that lead to endogenous production and release of  $H_2S$  are not known. In addition, the regulatory mechanisms controlling the expression of  $H_2S$ -producing enzymes are also largely unknown. Experimentally, estrogen (E2) may induce CSE expression through the estrogen receptor  $\alpha$ , which induces

the transcription of specificity protein-1 (SP1) (Wang et al. 2015b). However, various cell activators including phorbol-12-myristate-13-acetate, dibutyryl-cAMP, dexamethasone, or glucagon did not affect the transcriptional activity of CSE (Ishii et al. 2004). In mouse pancreatic cells, increases in glucose and intracellular  $\text{Ca}^{2+}$  levels can potentially induce CSE expression through activation of the Elk and/or SP1 transcription factors (Ishii et al. 2004; Taniguchi et al. 2012).

MicroRNAs (miRNAs) are small (~22 nucleotide), noncoding RNAs that are responsible for the regulation of mRNA and protein expression through translational inhibition or mRNA degradation (Bartel 2004). Recent studies have revealed the existence of an  $\text{H}_2\text{S}$ –miRNA crosstalk mechanism and that miRNA-mediated  $\text{H}_2\text{S}$  production via upregulated CSE gene expression may be connected to its amelioration of cardiac dysfunction (Shen et al. 2015). Diminished endogenous  $\text{H}_2\text{S}$  levels result in reduced tetrahydrobiopterin ( $\text{BH}_4$ ) levels, eNOS uncoupling, and profound oxidative stress (Polhemus and Lefer 2014). A complete genetic deficiency in CBS (i.e., a homozygous knockout mouse) is not necessarily embryonic lethal; however, developing embryos showed severe growth retardation and most infants eventually died within 5 weeks after birth (Watanabe et al. 1995). Only limited data are available describing the phenotype of CBS heterozygous knockouts (Polhemus and Lefer 2014; Watanabe et al. 1995). CSE $^{-/-}$  mice and CSE-overexpressing transgenic mice have been developed, and these models have fairly well-characterized the importance of CSE in terms of cardiovascular disease (Yang et al. 2008). In addition to the low abundance of CSE in infants, CSE activity can also be impaired in certain clinical conditions such as hepatic cirrhosis, sepsis, or surgical stress (Ishii et al. 2004). The third pathway for  $\text{H}_2\text{S}$  generation requires two enzymes: cysteine aminotransferase (CAT) and 3-mercapto-pyruvate sulfurtransferase (3-MST), which produce  $\text{H}_2\text{S}$  and pyruvate from 3-mercaptopyruvate, which in turn is formed from cysteine and  $\alpha$ -ketoglutarate produced by CAT. An additional biosynthetic pathway for endogenous  $\text{H}_2\text{S}$  production that operates in the cerebellum and kidneys has been also described, which involves D-cysteine, 3-MST, and D-amino acid oxidase (Shibuya et al. 2013). In the mitochondria,  $\text{H}_2\text{S}$  can be produced constitutively by 3-MST and CBS. With respect to the termination of  $\text{H}_2\text{S}$  signaling, mitochondrial involvement may contribute to its catabolism in peripheral tissues (Farrugia and Szurszewski 2014; Bouillaud and Blachier 2011), as  $\text{H}_2\text{S}$  can be biodegraded through a series of oxidations involving sulfide quinone oxidoreductase (SQR), persulfide dioxygenase (ETHE1), and rhodanese via hydropersulfides, sulfite, thiosulfate ( $\text{S}_2\text{O}_3^{2-}$ ), and sulfate ( $\text{SO}_4^{2-}$ ). Termination of  $\text{H}_2\text{S}$ -mediated signaling occurs non-enzymatically by  $\text{H}_2\text{S}$  trapping in sulfane–sulfur pools and bound-sulfate pools (Farrugia and Szurszewski 2014; Ishigami et al. 2009). Conversely, these sulfane–sulfur pools and bound-sulfate pools can also serve as sources of  $\text{H}_2\text{S}$  release. Thiosulfate produced from cysteine oxidation by cysteine dioxygenase, rather than from  $\text{H}_2\text{S}$ -derived thiosulfate produced in the mitochondria, is excreted in the urine and is considered to be a specific marker of whole-body  $\text{H}_2\text{S}$  production (Kamoun et al. 2003).

## 2.3 Nitric Oxide (NO)

NO has an unpaired electron and can become ionized in water (Table 1); as such, it can interact with different targets within cells. The half-life of NO in solution is less than a few seconds, and NO is quickly oxidized in non-enzymatic reactions with O<sub>2</sub> to form nitrite and nitrate (Motterlini and Foresti 2017). Nitrite per se is a physiologically inert NO metabolism end product, but may serve as an endocrine NO reservoir in tissues and blood (Nesci et al. 2016). Nitrite and nitrate are excreted by the kidneys (Kajimura et al. 2010). NO can bind either to ferrous (Fe<sup>2+</sup>, to which it has a higher affinity) or ferric (Fe<sup>3+</sup>) heme; it can also react with thiols to form S-nitrosothiols with superoxide to produce a potent oxidant peroxynitrite, and with tyrosine to form nitrosotyrosine. In red blood cells, NO binds covalently to a cysteine residue in the beta-chain of Hb and forms methemoglobin (MetHb, Hb [Fe]NO), a form of oxidized hemoglobin that does not bind oxygen. When a high level of MetHb is caused by either metabolic deficiencies in MetHb reductase or exposure to oxidizing medications such as dapsone (4,4'-diaminodiphenylsulfone), tissue O<sub>2</sub> deprivation can be caused by a decrease in the release of O<sub>2</sub> from O<sub>2</sub>-Hb (Kolluru et al. 2016; Ash-Bernal et al. 2004). In contrast, S-nitrosohemoglobin (SNO-Hb) can be formed from NO-bound Hb via an oxygenation-induced allosteric transition in Hb (McMahon et al. 2005; Burger et al. 2009). The biological role of SNO-Hb has been studied from the point of view of SNO-Hb-mediated hypoxic vasodilation (Allen et al. 2009; Hare et al. 2013; Riccio et al. 2016).

Endogenous NO is produced by NOS, which is a heme protein composed of an oxygenase and a reductase domain (Kajimura et al. 2010). NOS requires the amino acid L-arginine and oxygen, along with the reducing agent NADPH for activity (Fig. 1). Unlike O<sub>2</sub>, the superoxide ion (O<sub>2</sub><sup>-</sup>) can reduce the bioavailability of NO, affecting either NO biosynthesis by reducing L-arginine levels, or by suppressing the enzymatic activity of NOS (Sjoberg and Singer 2013). In contrast to HO, enzymatically generated NO can autoregulate NOS activity, as NO can bind to heme-containing proteins in both the Fe<sup>2+</sup> and Fe<sup>3+</sup> oxidation states (Griscavage et al. 1994). HO requires NADPH and O<sub>2</sub> to produce CO, whereas NOS requires an additional cofactor such as BH<sub>4</sub>, flavin adenine dinucleotide, or flavin mononucleotide for NO production. Three NOS isoforms exist and are known as NOS1 (neuronal, nNOS), NOS2 (inducible, iNOS), and NOS3 (eNOS). Both nNOS and eNOS are constitutively expressed, but can be further activated in response to CaM, and their specificities are further controlled by specific subcellular domains (Mustafa et al. 2009a). The cellular contexts of NOSs is of primary importance for producing NO, although the bioavailability of BH<sub>4</sub> in CVS is also important for NO production (Bendall et al. 2014) as BH<sub>4</sub> appears essential for electron donation to the heme group in the oxygenase domain to oxidize L-arginine in all three NOSs; however, its oxidation/reduction status is highly sensitive to oxidative stress. Therefore, abnormalities in the absolute levels and oxidation state of BH<sub>4</sub> are a hallmark of vascular and cardiac dysfunction caused by impaired NO production and imbalanced NO and O<sub>2</sub><sup>-</sup> synthesis by eNOS (comprehensively reviewed in Bendall et al. 2014).



In the heart, the major source of NO released into the coronary microvasculature is eNOS, which is predominantly located in the coronary and cardiac endothelium (Pacher et al. 2007). NO is also produced by cardiac myocytes from eNOS located in cell membrane caveolae and from nNOS, which co-localizes with ryanodine receptors (RyR2) in the sarcoplasmic reticulum (Barouch et al. 2002). Recently, the existence of a still-putative mitochondrial NOS (mtNOS) was proposed (Kanai et al. 2004). It has been argued that the amounts of NO produced by non-mitochondrial nNOS under physiological conditions is insufficient for regulating mitochondrial respiration during  $\beta$ -adrenergic stimulation, countering intracrine control of respiration by NO in cardiac myocytes (Kohlhaas et al. 2017). eNOS $^{-/-}$  mice were observed to be physically and histologically similar with wild-type mice, except that they exhibited lower body weights. However, increased blood pressures and reduced heart rates were found in eNOS $^{-/-}$  mice (Shesely et al. 1996). Apart from changes in the protein-expression levels of distinct NOS isoforms, various protein interactions and post-translational modifications (such as protein phosphorylation and lipid modification) can modify their activities to control their activation in response to upstream signal-transduction events (Bredt 2003). In red blood cells, NO can be produced non-enzymatically from nitrite ( $\text{NO}^{2-}$ ) by deoxyhemoglobin, which acts as a nitrite reductase. Circulating nitrate ( $\text{NO}^{3-}$ ) in the blood can be secreted through the saliva glands and reduced to nitrite. Maintaining the amount of nitrite in the circulation and in tissues serves as a buffer against hypoxia, which can decrease thrombosis and promote vasodilation (Webb et al. 2008). Nitrite reduction to NO (or to other important metabolites) occurs in mitochondria. Nitrite might have difficulty in diffusing freely across the mitochondrial membrane (Sharma et al. 1999), considering that only approximately 10% of the nitrite incorporated in isolated mitochondria is internalized (Nesci et al. 2016).

### 3 Cellular Regulatory Roles of Gasotransmitters: Mechanistic Considerations

Based on their dipole moments and water solubilities (Table 1), CO, H<sub>2</sub>S, and NO are highly membrane-permeable (Kajimura et al. 2010) and are not readily stored in vesicular structures (as are hormones and other neurotransmitters); thus, they must be synthesized as needed (Mustafa et al. 2009a). Although sGC can serve as a cytosolic receptor for NO (Martin et al. 2005), the receptors for specific gasotransmitters are poorly defined, which leads to limited understating of their specific regulatory roles in signal-transduction pathways. However, their rapid signaling effects, at least in part, on ion channels (Fig. 2 and Table 2) and mitochondrial respiration (Fig. 3) strongly suggest that the associated biosynthetic enzymes are subject to extraordinarily nuanced regulatory mechanisms. The underlying molecular regulatory mechanisms involved in the timely production of each gasotransmitter should be further clarified in the near future. Once produced, each gasotransmitter



exerts its biological functions through various macromolecular interactions including the covalent binding of gases to prosthetic metal complexes in acceptor proteins, non-covalent binding to protein regulatory subunits, and space occupancy in and around protein structures, which obstructs the access of other gases to functionally important protein motifs (Takahashi et al. 2012; Mustafa et al. 2009a).

### 3.1 *Post-translational Modifications*

CO, H<sub>2</sub>S, and NO can post-translationally modify the amino acid moieties of proteins to either inhibit or stimulate their activities (Kyle and Braun 2014; Stram and Payne 2016; Victorino et al. 2015). CO can exert its biological actions by activating guanylate cyclase (GC) and/or directly carbonylating its proline, threonine, lysine, and arginine residues, whose modifications are not very sensitive to reducing agents (Cattaruzza and Hecker 2008; Retamal 2016), or through the indirect cysteine residue carbonylation in a lipid peroxidation-dependent process (Wong et al. 2013). Previously, it was suggested that H<sub>2</sub>S can lead to protein S-sulfhydration (persulfidation) by covalently converting the –SH group of cysteine to an –SSH group in the protein (Mustafa et al. 2009b), thereby increasing the activities of various enzymes (Vandiver and Snyder 2012; Shintani et al. 2009; Sen 2017), changing the localization and stability of proteins inside cells, and increasing the resistance of proteins to oxidative stress (Sen 2017; Paul and Snyder 2012; Modis et al. 2016). However, this manner of sulfhydration is debated because thiols and H<sub>2</sub>S will not react with each other if they have the same oxidation state (Kimura 2016). Cysteine sulfhydration has been proposed to occur via reaction with hydrogen polysulfide under normal cellular conditions, with oxidized thiols under oxidative conditions, and/or following NO signaling via reaction with H<sub>2</sub>S (Kimura 2016). For example, sulfhydration of the ATP-sensitive potassium channel (K<sub>ATP</sub>) inhibits ATP binding, but enhances phosphatidylinositol-4,5-bisphosphate binding, which results in channel opening and the vasodilation of smooth muscle cells (Sen 2017). The chemical reactivity of NO is well-suited for interactions with the sulfhydryl group in cysteine residues; therefore, S-nitrosylation of numerous proteins occurs in the vasculature and the nervous system (Anand and Stamler 2012). However, S-nitrosylation is extremely labile; thus, it is more challenging to identify proteins that are physiologically nitrosylated under basal conditions (Mustafa et al. 2009a). The NO-cyclic GMP (cGMP) pathway is a canonical mediator of NO signaling (Francis et al. 2010). More recently, the role of a cGMP-independent signaling pathway involving protein S-nitrosylation has been increasingly realized (Takahashi et al. 2012). Multiple enzymes; e.g., nitrosylases and denitrosylases, have been identified in physiological settings, which participate in targeted S-nitrosylation (addition of an NO group by Cys-to-Cys transfer or metal-to-Cys transfer) or protein denitrosylation (removal of the NO group from SNO-modified proteins) (Anand and Stamler 2012). The redox state of the cell heavily depends on the balance between nitrosylation and denitrosylation, thus establishing fundamental

crosstalk between NO and reactive oxygen species (ROS) (Semenza and Prabhakar 2012).

Apart from protein modifications caused by a single gasotransmitter, combined effects mediated by various pro-oxidants and each gasotransmitter acting on the same protein are known to occur (Wilkinson and Kemp 2011), considering that proteins in the body are not strictly exposed to single gasotransmitters, as investigated in experimental settings. For example, it is not clear whether the same cysteine residues that are S-nitrosylated also undergo disulfide bond formation, or whether proteins that are nitrated can be sulfhydrated, and how these different redox modulations affect protein functions (Akbarali and Kang 2015). Thus, sequential post-translational modifications and/or the functional hierarchies of modifications with gasotransmitters should be studied in the future to better understand their functional consequences.

### ***3.2 Binding to Prosthetic Metal Complexes in Acceptor Proteins***

In mammals, a large number of heme-containing proteins function as receptors, sensors, or transporters of gaseous molecules (e.g., hemoglobin and myoglobin), including O<sub>2</sub>, CO, H<sub>2</sub>S, and NO (Motterlini and Foresti 2017; Wegiel et al. 2014). Importantly, two fundamental factors determine the affinity and selectivity of heme-dependent proteins for gaseous molecules. The first is the intrinsic reactivity of ferrous heme with diatomic gases. The ferrous form (Fe<sup>2+</sup>) of hemoglobin preferentially binds ligands such as CO, NO, and O<sub>2</sub>. In contrast, ferric heme (Fe<sup>3+</sup>) preferentially binds water, H<sub>2</sub>S, and anions such as the cyanide ion (CN<sup>-</sup>), N<sub>3</sub><sup>-</sup>, and OH<sup>-</sup> (Kajimura et al. 2010). The second factor constitutes the ligand geometry and chemistry on the distal side of the heme pocket (Tsai et al. 2012). Together with steric constraints and electrostatic interactions of the bound ligand on the distal side of the heme molecule, the aforementioned factors can profoundly affect the selectivity of heme toward gaseous molecules (Tsai et al. 2012). The binding of CO to a transition metal in a specific redox state; e.g., Fe<sup>2+</sup>-heme, is a possible type of chemical interaction that can occur within biological systems (Cebova et al. 2016; Motterlini and Foresti 2017). CO can compete with O<sub>2</sub> for binding to the four heme iron centers in Hb to form carboxy-Hb (Rochette et al. 2013). CO affinity for metal atoms allows for its interaction with and modification of the activities of a range of metalloproteins, such as a Hb (inhibition), myoglobin (inhibition), sGC (activation), iNOS (inhibition), cyclooxygenase (activation or inhibition), cytochrome p450 (inhibition), cytochrome c oxidase (inhibition), and NADPH: oxidase (inhibition), as well as others (reviewed in Wu and Wang 2005). This characteristic implicates all heme-containing proteins as possible biological CO sensors, despite the fact that they have other physiological ligands (Kim et al. 2006). In contrast to CO and NO, H<sub>2</sub>S seems incapable of stimulating sGC, even though it can bind with reasonably

high affinity to heme-containing domains, such as those exhibited in sGC. With respect to transcriptional regulation, CO can also regulate gene expression via heme-binding transcription factors such as BACH1 and NPAS2 (Wilkinson and Kemp 2011; Dioum et al. 2002). However, several other proteins that do not contain heme groups or transition metals, such as MAPKs, can be modulated by CO, presumably by indirect mechanisms (Wilkinson and Kemp 2011). In addition to directly modulating signal transduction through S-nitrosylation of proteins such as arginase, G protein-coupled receptor kinases, the ryanodine receptor (RyR), and numerous other proteins (Lundberg et al. 2015), NO can also participate in regulating gene expression. Recently, it was shown that transcriptional repression mediated by the heme-bound nuclear receptor REV-ERB (Benoit et al. 2006) could be reversed by NO and that the effects of heme and NO were mediated by the C-terminal ligand-binding domain of REV-ERB (Pardee et al. 2009). The instantaneous response of REV-ERB proteins to NO signaling and the significance of these gas- and REV-ERB-regulated physiological mechanisms suggest the prospect of novel, gas-based therapies in treating related diseases such as atherosclerosis, depression, diabetes, mood- and sleep-based disorders, obesity, and osteoarthritis (Pardee et al. 2009). Furthermore, angiogenesis requires the promotion of cell survival and proliferation and is controlled through gene regulation. As both microvessel formation and wound healing were impaired in CSE<sup>-/-</sup> mice (Papapetropoulos et al. 2009), this finding suggests that H<sub>2</sub>S may be involved in the genetic control required for angiogenesis, but although its underlying mechanism(s) has not been clearly identified. Collectively, previous data have indicated that CO, H<sub>2</sub>S, and NO can bind the heme moiety in proteins, leading to conformational changes that either activate or inhibit heme-containing proteins to differing extents, depending on the protein.

## 4 Modulation of Ion Channels by CO, H<sub>2</sub>S, and NO

Proper heart function is strongly dependent on a finely tuned balance between all cardiac ion channels producing the myocardial action potential (AP); thus, impairment of any of these ion channels could cause severe problems in heart hemodynamics. Peripheral arteriolar contraction is tonic (tone-maintaining), whereas cardiac contraction is short-lived and generates considerable force, owing to differences in controlling cytosolic calcium levels and generating contractile forces (Roden et al. 2002). The contractile mechanism in vascular smooth muscle tissues differs in several fundamental ways from that of the myocardium, including a different signal linking cytosolic calcium to muscle contraction; namely, the activation of myosin light chain kinase by CaM. The cAMP formed in response to  $\beta$ -agonist stimulation has a vasodilatory effect, thereby inhibiting contraction, in contrast to the stimulation of contraction achieved in the myocardium. Increased cGMP levels occur not only via CO and NO, which lead to sGC activation, but also via H<sub>2</sub>S, which inhibits cGMP phosphodiesterase (PDE) activity (Fig. 2). The PKG can then phosphorylate and directly affect the activity of channels such as the L-type Ca<sup>2+</sup> channel, K<sub>ATP</sub>,

$\text{Ca}^{2+}$  activated ( $\text{K}_{\text{Ca}}$ ) potassium channel, and the large-conductance calcium-activated ( $\text{BK}_{\text{Ca}}$ ) potassium channel (Francis et al. 2010). However, the sGC–cGMP–PKG pathways are very diverse and too complex to be properly addressed here (Papapetropoulos et al. 2015a; Francis et al. 2010; Hoffmann and Chen 2014); thus, changes in channel activities mediated by PKG are not discussed further in this review.

Ion channels comprise central molecules mediating signal transduction across biological membranes. Owing to the easy accessibility and relatively low expression in specific cells and tissues, some ion channels constitute important drug targets for channel-associated human diseases caused by either genetic or acquired factors (e.g., channelopathies) (Kim 2014; Amin et al. 2010; Campuzano et al. 2010). Furthermore, it has been suggested that dysfunctions in the complex interactions between ion channels and other proteins can result in serious diseases, such as diabetes and heart-related diseases (Niemeyer et al. 2001). Many ion channels in the CVS have yet to be investigated in terms of their sensitivity to CO,  $\text{H}_2\text{S}$ , and NO (Cebova et al. 2016; Roden et al. 2002; Catterall 2000; Shieh et al. 2000; Algalarrondo and Nattel 2016; Fernandez-Falgueras et al. 2017; Yu et al. 2017a). These gasotransmitters modulate different channels through a variety of different mechanisms, which include the post-translational modification of ion channel residues, as well as direct or indirect heme binding (Table 2). The tissue- and/or species-specific expression of certain ion channels may cause various effects, even at the same gasotransmitter concentration. Therefore, the underlying mechanisms of gasotransmitters involved in regulating ion channels, as well as their impacts under physiological and/or pathological conditions, should be further defined.

## 4.1 Potassium ( $\text{K}^+$ ) Channels

The potassium ( $\text{K}^+$ ) channel (Akrouh et al. 2009; Humphries and Dart 2015) is a hetero-octameric structure consisting of four pore-forming subunits (Kir6.x) and four regulatory sulfonylurea receptors (SURx).  $\text{K}^+$  channels gate very quickly, have complex inactivation kinetics, and can be subject to elaborate regulation by voltage, as well as intracellular and extracellular ion concentrations.  $\text{K}^+$  channels play an essential role in maintaining and stabilizing the resting membrane potential. The opening of  $\text{K}^+$  channels leads almost universally to  $\text{K}^+$  efflux from the cell, causing the membrane potential to become more negative (Humphries and Dart 2015). In addition,  $\text{K}^+$  regulates caspase activation and the mitochondrial volume and membrane potential (Dallas et al. 2011). Therefore,  $\text{K}^+$  channels occupy distinct physiological niches within the human body (Humphries and Dart 2015). Among  $\text{K}^+$  channels, the  $\text{K}_{\text{ATP}}$  channels are expressed on the cell surface and, notably, in the mitochondria, where they are potential targets for cardioprotection (Roden et al. 2002). Although the exact mechanisms whereby CO and  $\text{H}_2\text{S}$  modulate all  $\text{K}^+$  channels have not been well characterized (Table 2), the  $\text{K}_{\text{ATP}}$  channel is activated through direct interactions with CO and  $\text{H}_2\text{S}$ , which leads to vasorelaxation of the

vasculature (Soni et al. 2010) and cardioprotection against I/R injury (Tang et al. 2005; Jaggar et al. 2005). Under physiological conditions, activation of the  $K_{ATP}$  channel by exogenous  $H_2S$  to some extent causes negative inotropic/chronotropic effects in the heart (Geng et al. 2004a).  $H_2S$  generated from VSMCs can relax vascular tissues through direct activation of  $K_{ATP}$  channel without the involvement of second messengers, such as cGMP, cAMP, and protein kinase C (PKC) pathways (Wang 2003; Tang et al. 2010). This activation of  $K_{ATP}$  channels by  $H_2S$  is associated with sulfhydrylation of its channel moiety, which causes either reduced ATP binding or enhanced  $PIP_2$  binding to the Kir 6.1 subunit (Mustafa et al. 2011). NO can activate  $K_{ATP}$  channels in DRG neurons through direct S-nitrosylation of the SUR1 subunit. When Cys717 in the nucleotide-binding domain 1 of SUR1 was mutated, the channel showed decreased current even in the presence of NO donors (Kawano et al. 2009). Compared to the actions of  $H_2S$  and NO on the  $K_{ATP}$  channel, NaHS (an  $H_2S$  donor) significantly enhanced SUR2B sulfhydrylation, whereas SIN-1 led to ONOO<sup>-</sup> generation from NO and Kir6.1 subunit superoxide-induced nitration (Akbarali and Kang 2015). Moreover, sulfhydrylation of one subunit (SUR2B) of the  $K_{ATP}$  channel hinders tyrosine nitration in the other subunit (Kir6.1). In addition, NaHS also reversed ONOO<sup>-</sup>-induced inhibition of smooth muscle contraction (Akbarali and Kang 2015). Because tyrosine nitration of Kir6.1 is unaffected by NaHS, it appears that the sequence of exposure to  $H_2S$  or NO may be critical in regulating  $K_{ATP}$  channel activity (Akbarali and Kang 2015). Although the exact mechanism by which CO modulates  $K_{ATP}$  activation is not well established, its cardioprotective effect against I/R injury is associated with  $K_{ATP}$  channel activation, considering that CO-releasing molecule-2 (CORM-2)-mediated reduction in I/R injury was abolished in the presence of the  $K_{ATP}$  channel inhibitor glibenclamide (Soni et al. 2010).

BK<sub>Ca</sub> potassium channels are comprised of pore-forming  $\alpha$  subunits, as well as accessory  $\beta$  subunits that modulate channel gating (Rothberg 2012). This channel provides a key physiological link between  $Ca^{2+}$  signaling and electrical signaling at the plasma membrane to regulate the smooth muscle tone of resistance arteries. BK<sub>Ca</sub> channel activity is regulated by various signaling molecules, such as intracellular  $Ca^{2+}$ , protein kinases, tyrosine kinases, heme, and cytochrome P-450-derived metabolites of arachidonic acid (Rothberg 2012). BK<sub>Ca</sub> channels are also activated by physiologically relevant gases including CO through heme binding (Jaggar et al. 2005; Rothberg 2012; Dong et al. 2007; Wang et al. 1997),  $H_2S$  through persulfidation (Mustafa et al. 2011), and NO through sulfhydryl modification (Bolotina et al. 1994) in a cGMP-PKG-independent mode (Fig. 2 and Table 2). In contrast, CO and NO (which can both activate sGC) and  $H_2S$  (which can suppress the activity of the cGMP-degrading PDE) are also involved in activating BK<sub>Ca</sub> in a cGMP-PKG-dependent manner (Fig. 2). It is unclear what determines whether BK<sub>Ca</sub> is activated in a cGMP-independent mode or a cGMP-dependent mode, following exposure to CO,  $H_2S$ , or NO. One hypothesis is that the individual concentrations of CO,  $H_2S$ , and NO, and the time gap between direct modulation of BK<sub>Ca</sub> by these gasotransmitters and indirect modulation through the sGC-cGMP-PKG-BK<sub>Ca</sub> pathway influence BK<sub>Ca</sub> activation. Other subtypes of calcium activated  $K^+$  channels, as

well as intermediate-conductance ( $IK_{Ca}$ ) and small-conductance ( $SK_{Ca}$ ) potassium channels can be activated by  $H_2S$ -mediated cysteine S-sulphydration (Mustafa et al. 2011). Regarding  $BK_{Ca}$  activation by CO, it has been suggested that the interaction of heme with  $BK_{Ca}$  could modulate its channel activity (Rothberg 2012). Notably, the  $BK_{Ca}$  channel does not usually possess a transition metal, although heme- $BK_{Ca}$  complexes can form through binding to heme derived either from hemeproteins or endogenous heme-like molecules, and the formation of activated  $BK_{Ca}$  alters the sensitivity of the channel to allosteric ligands (Tang et al. 2003). In this model, CO binds to heme, after which the complex can bind to  $BK_{Ca}$  channels to regulate their activity (Jaggar et al. 2005). Evidence for this model comes from the observations that hemin and NADPH (both HO substrates) resembled the effects of exogenously added CO when applied to excised membrane patches embedded with  $K^+$  channels, whereas these effects were reversed by HO inhibitors. Conceivably, HO activation in the proximity of the  $BK_{Ca}$  channel may not only increase the levels of CO (a  $BK_{Ca}$  channel activator), but may also reduce the levels of membrane-associated heme acting as a  $BK_{Ca}$  channel inhibitor (Jaggar et al. 2005). Through the inhibitory action of CO at the  $BK_{Ca}$  channel (Kim et al. 2006; Williams et al. 2004), the development of hypoxic pulmonary artery hypertension can potentially be attenuated by exogenous CO supplementation.

## 4.2 Calcium ( $Ca^{2+}$ ) Channels

Found in numerous cell types,  $Ca^{2+}$  channels are activated upon membrane depolarization and are responsible for mediating  $Ca^{2+}$  influx owing to action potentials and sub-threshold depolarizing signals (Catterall 2000; Keef et al. 2001). In cardiac, skeletal, and smooth muscle tissues, the major  $Ca^{2+}$  currents are characterized by a high activation voltage, large single-channel conductance, slow voltage-dependent inactivation, a cAMP-dependent protein phosphorylation regulatory pathway, and inhibition by specific  $Ca^{2+}$ -antagonist drugs (Catterall 2000; McCleskey et al. 1986). Among the voltage-gated  $Ca^{2+}$  channels, the L-type  $Ca^{2+}$  channel (LTCC) is a plasma membrane-associated hetero-tetramer complex, comprised of an  $\alpha 1$  subunit ( $\approx 240$  kDa), a  $\beta 2$  subunit ( $\approx 60$  kDa), and an  $\alpha 2\text{-}\delta$  subunit ( $\approx 175$  kDa) subtyped as  $Ca_v 1.2$  (Catterall 2000). The LTCC is inactivated after CaM binds to the C-terminal domain of the  $Ca_v 1.2$  channel. When the LTCC is suppressed, both the heart rate and atrioventricular node conduction are decreased, which leads to cardiac vasodilation. Activation of LTCCs by CO has been reported in ventricular myocytes in 4-week-old pups born from CO-exposed (150 ppm) rats, although this effect was not seen at 2 or 8 weeks (Sartiani et al. 2004). Conversely, CO (applied as a dissolved gas or via a recognized CO donor molecule) caused reversible, voltage-independent channel inhibition associated with ROS generation by complex III in mitochondria (Scragg et al. 2008). The leading cause of cell death in post-ischemic cardiomyocytes is calcium overload, through either apoptosis or necrosis, and LTCC suppression by CO plays a role in reducing I/R injury. In addition to the PKG,

cAMP-dependent protein kinase (PKA), and PKC pathways, LTCC activity can be modulated by other protein tyrosine kinases such as a c-Src kinase and CaM-dependent kinase (Keef et al. 2001). When tyrosine nitration of the LTCC occurs via peroxynitrite, LTCC phosphorylation by c-Src kinase is suppressed and results in a decrease in its channel activity (Keef et al. 2001; Campbell et al. 1996). Compared to tyrosine nitration, S-nitrosylation by S-nitrosothiols (which donate NO) stimulates LTCCs in ventricular myocytes (Campbell et al. 1996). H<sub>2</sub>S delivered as NaHS has also been shown to inhibit LTCCs (Avanzato et al. 2014).

The low-voltage-activated T-type Ca<sup>2+</sup> channels, referred to as Ca<sub>v</sub>3.1–Ca<sub>v</sub>3.3, open during membrane depolarization (Elies et al. 2016; Nilius and Carbone 2014; Talavera and Nilius 2006; Nilius et al. 1985). In neurons, the T-type calcium channel Ca<sub>v</sub>3.2 is inhibited not by direct interaction with CO, but rather by disruption of thioredoxin regulation, which acts as a tonic, endogenous regulator of the T-type Ca<sub>v</sub>3.2 channel at an extracellular site (Boycott et al. 2013). Although Ca<sub>v</sub>3.1 and Ca<sub>v</sub>3.2 are expressed in VSMCs, they do not exhibit classic low-voltage-activated calcium currents, owing to splice variations (Kuo et al. 2014). Aberrant activation of Ca<sub>v</sub>3.1 causes pulmonary hypertension and the associated vascular hyper-reactivity, pulmonary arterial remodeling, and right cardiac hypertrophy (Chevalier et al. 2014). H<sub>2</sub>S can selectively inhibit Ca<sub>v</sub>3.2, but both Ca<sub>v</sub>3.1 and Ca<sub>v</sub>3.3 are less sensitive to H<sub>2</sub>S exposure in dorsal root ganglion neurons (Elies et al. 2014). In rat cardiomyoblasts, H<sub>2</sub>S has also been shown to inhibit T-type Ca<sup>2+</sup> channels through an unidentified mechanism (Avanzato et al. 2014). In the CVS, the direct involvement of CO, H<sub>2</sub>S, and NO in modulating T-type Ca<sup>2+</sup> channel activity has not been well defined (Elies et al. 2016; Okubo et al. 2011; Takahashi et al. 2013). Moreover, critical roles of T-type calcium channels involved in the CVS and their pathological impacts should be more clearly identified to better understand the physiological effects of H<sub>2</sub>S or other gasotransmitters.

### **4.3 Transient Receptor Potential Ion Channels**

Transient receptor potential (TRP) ion channels comprise a family of proteins characterized by six membrane-spanning domains and are permeable to monovalent cations and calcium ions (Harteneck et al. 2000). TRP channels can sense several gasotransmitters, such as NO, O<sub>2</sub>, H<sub>2</sub>S, and CO<sub>2</sub> (Takahashi et al. 2008). Gasotransmitter-responsive TRP ion channels implicated in the neuronal system have been increasingly found in the CVS (Takahashi et al. 2012; Shimizu et al. 2014; Kozai et al. 2015; Andrei et al. 2016; Alonso-Carbajo et al. 2017). The TRP ankyrin 1 (TRPA1) channel, present in the endothelium, can elicit dilation of arteries through the nerve-mediated release of G protein coupled receptors or via endothelium-dependent smooth muscle cell hyperpolarization (Earley 2012). NO can either activate TRP channels via NO-mediated cysteine S-nitrosylation (Kozai et al. 2015; Yoshida et al. 2006) or suppress TRPC6 activity via cGMP-PKG dependent phosphorylation (Takahashi et al. 2008). Studying H<sub>2</sub>S-mediated



TRPA1 activation is challenging because only supra-physiological concentrations of  $\text{H}_2\text{S}$  can activate TRPA1 (Eberhardt et al. 2014) and NO-dependent cysteine-S-nitrosylation is quite slow (Lancaster 2006). The recent observation of TRPA1 activation in the presence of  $\text{H}_2\text{S}$  and/or NO at physiological concentrations was suggested to result from HNO, which is chemically generated by reaction between NO and  $\text{H}_2\text{S}$  and induces the formation of amino-terminal disulfide bonds on TRPA1 in sensory neurons (Eberhardt et al. 2014). Polysulfides ( $\text{H}_2\text{S}_n$ ) such as  $\text{H}_2\text{S}_2$  and  $\text{H}_2\text{S}_3$ , which are chemically generated by chemical reactions between  $\text{H}_2\text{S}$  and NO, contribute to TRPA1 activation in a cyanolysis-resistant manner (Miyamoto et al. 2017). Therefore, the regulatory roles of NO,  $\text{H}_2\text{S}$ , and their chemically formed secondary metabolites in the CVS will likely be studied extensively to reveal their direct influences on cardiovascular functions, as well as their indirect control of the CVS (initiated by sensory neurons).

#### 4.4 Hemi-Channels

Hemi-channels are comprised of six connexin (Cx) monomers and are molecularly permeable with masses of up to 1.2 kDa in size. These undocked hemi-channels contribute to physiological autocrine/paracrine cell signaling, the spreading of calcium waves, and  $\text{Ca}^{2+}$  permeation across the plasma membrane. Although the regulatory roles of CO,  $\text{H}_2\text{S}$ , and NO on hemi-channels have not been well established, these hemi-channels might be direct targets for these gasotransmitters. For example, CO could increase free radical concentrations, which could then induce lipid peroxide production. In turn, these could trigger extracellular cysteine carbonylation (via their  $-\text{SH}$  groups) to increase the  $\text{Ca}^{2+}$  sensitivity of Cx46, thereby stabilizing the closed state of loop gating in Cx46 (Retamal 2016). Homeostatic regulation of the partial pressure of  $\text{CO}_2$  ( $\text{PCO}_2$ ) is extremely important in maintaining vitality. Recently, it has been reported that  $\text{CO}_2$  can directly modulate the activity of CX26 through formation of carbamate bridges between subunits (Meigh et al. 2013).

### 5 Regulatory Roles of CO, $\text{H}_2\text{S}$ , and NO in the Mitochondria

#### 5.1 Mitochondrial Bioenergetics and Cardioprotection

The rhythmic contraction of the heart ensures oxygenation in the organs of the body, and its function is dependent on mitochondrial aerobic energy delivery via the electron-transport chain (ETC) and the oxidative-phosphorylation system (OXPHOS) (Chaudhary et al. 2011). In addition, biological functions such as ATP



delivery, heat production, metal homeostasis, stress signaling, and defense responses are coordinated within mitochondria (Kaniak-Golik and Skoneczna 2015; da Cunha et al. 2015; McBride et al. 2006; Picard et al. 2016). Mitochondrial  $\text{Ca}^{2+}$  release and uptake are largely dependent on the status of the mitochondrial membrane potential (Burwell and Brookes 2008). Aberrant respiratory chain inhibition leads to drastic changes in mitochondrial  $\text{Ca}^{2+}$  homeostasis, which can be aggravated by mitochondrial-permeability transition pore (MPTP) opening (Di Lisa et al. 2007; Di Lisa and Bernardi 2015). Thus, it is not surprising that mitochondrial diseases mainly affect the heart, because impaired cardiac conditions are associated with mitochondrial dysfunction and originate from defects in either ETC or OXPHOS (Schwarz et al. 2014; Finsterer and Kothari 2014; Lee et al. 2016; Vasquez-Trincado et al. 2016). One causal factor in aberrant mitochondrial respiration is a dysregulation in the level of oxygen, which serves as the primary electron acceptor in several intracellular biochemical reactions including ATP generation by mitochondria. High or low oxygen levels can promote stress, depending on the physiological or biological context. Low oxygen levels (hypoxia) in tissues can result from several pathophysiological conditions including atherosclerosis, cancer, chronic obstructive pulmonary disease, diabetes, inflammatory diseases, ischemic disorders (cerebral or cardiovascular), pre-eclampsia, or psoriasis (Brahimi-Horn and Pouyssegur 2007; Ho et al. 2012).

Indeed, proteins containing mitochondrial heme are crucial for normal functioning of the mitochondria, such as oxidative phosphorylation and mitochondrial biogenesis. Oxygen-sensitive accumulation and degradation of heme proteins in mitochondria affects mitochondrial functions including bioenergetics and oxygen-sensing processes (Teng et al. 2013). Mitochondria are intimately involved in iron metabolism, from iron trafficking to heme synthesis and iron–sulfur cluster biogenesis (Paul et al. 2017). The Lon protease constitutes an important degradation enzyme found in the mitochondrial matrix (Koppen and Langer 2007). In particular, mitochondrial CBS and HO-1 can be degraded by the Lon protease depending on the presence of an oxygenated heme group in CBS or HO-1 (Teng et al. 2013). Ischemia or hypoxia can therefore lead to CBS and HO-1 accumulation in mitochondria if the Lon protease fails to efficiently degrade CBS and HO-1, owing to the transition of the heme moiety from an oxygenated to a deoxygenated form. When oxygen tension recovers during reperfusion, CBS and HO-1 can be readily degraded by the mitochondrial Lon protease. This aspect of Lon-mediated CBS and HO-1 regulation, which occurs in mitochondria, can fine-tune the local production of  $\text{H}_2\text{S}$  and CO, based on the  $\text{O}_2$  tension, thereby modulating mitochondrial heme metabolism. CO,  $\text{H}_2\text{S}$ , and NO can affect mitochondrial heme metabolism owing to their heme-binding capacities (Kajimura et al. 2010). The consequence of the regulation of mitochondrial heme metabolism by CO,  $\text{H}_2\text{S}$ , or NO may be important for understanding the critical roles of these gaseous molecules, which presently remain unclear (Paul et al. 2017).

It has been proposed that the reversal of endogenous inhibition or the wash-out of respiratory inhibitors during reperfusion may provide protection by allowing the gradual re-activation of mitochondrial function, avoiding sudden ROS and  $\text{Ca}^{2+}$

overload (Burwell et al. 2009). CO, H<sub>2</sub>S, and NO might also be involved in acute O<sub>2</sub> sensing; however, there is no evidence supporting their use of the mitochondrial ROS produced during OXPHOS for O<sub>2</sub> sensing (Kajimura et al. 2010). CO, H<sub>2</sub>S, and NO may also be involved in cardioprotection against I/R injury in a concerted or distinct manner. In the heart, oxygen consumption is controlled by NO originating from nNOS (but not eNOS), which is regulated through paracrine mechanisms. nNOS constitutes the most significant source of NO in cardiac myocytes under physiological conditions; however, nNOS is not localized in mitochondria and does not control respiration. Therefore, eNOS may control cardiac O<sub>2</sub> consumption in a paracrine manner, rather than through intracrine signaling (Kohlhaas et al. 2017). H<sub>2</sub>S and NO may act in concert to protect the heart against ischemic injury. Inhibition of NO production with L-NAME, a nonselective inhibitor of NO synthase, significantly attenuated the cardioprotective effects of H<sub>2</sub>S preconditioning (Nagpure and Bian 2016). Although these beneficial roles of gasotransmitters associated with reversible mitochondrial inhibition in cardiac I/R injury may be promising, the use of mitochondrial inhibitors causes very severe side effects (such as Parkinson's disease and Huntington's disease-like syndromes) in animals (Burwell et al. 2009). In this section, the cardioprotective roles of CO, H<sub>2</sub>S, and NO against cardiac I/R injury are briefly addressed, in view of their regulatory roles at the mitochondrial respiratory complex (Fig. 3).

Complex I (NADH ubiquinone oxidoreductase) serves as an important electron entry site into the respiratory chain in cardiac mitochondria. It is also critical for ROS generation (Qian and Matson 2017) and functions as an NADH/NAD<sup>+</sup> redox balance modulator (Di Lisa and Bernardi 2015). During hypoxia, there is an early onset of complex I defects (caused by decreased NADH dehydrogenase components); moreover, as ischemia progresses, further damages are incurred to include complex III by functional inactivation of the iron–sulfur protein (ISP) subunit and complex IV, owing to a selective decrease in its cardiolipin content, which is involved in conserving mitochondrial cytochrome c (Chen et al. 2007). It has been suggested that mitochondrial complex I can exist in two interconvertible states, either in an active (A) conformation (>85%) or in an inactive, dormant (D) conformation (5–15%), based on the enzyme-activation rate by pH and divalent cations (Ca<sup>2+</sup> or Mg<sup>2+</sup>) and D-form sensitivity to SH-reagents (Babot et al. 2014). The A–D transition of complex I may serve as a mechanism whereby the enzyme activity is fine-tuned (Drose et al. 2016). Through the A–D transition of complex I, mitochondria can respond to oxygen deprivation, and regulation of the complex I re-activation rate can ameliorate I/R damage (Babot et al. 2014). Complex I inhibition can directly inhibit the opening of MPTP through an increased NADH/NAD<sup>+</sup> ratio, which is critical in determining the open conformation state of the MPTP (Di Lisa and Bernardi 2015). An irreversible inhibitor of complex I, rotenone, is characterized by its high-affinity, and markedly attenuates ischemic damage to cardiolipin, cytochrome c, and cytochrome oxidase, even after prolonged ischemia (Chen et al. 2007). Inhibitors of complex I such as amobarbital, rotenone, and S-nitrosothiols can afford cardioprotection. Other kinds of reagents with diverse structures, including the anti-anginal agent ranolazine, analgesics such as capsaicin,

redox-cycling agents (menadione), volatile anesthetics (halothane, isoflurane), and anti-diabetic drugs (such as metformin) can provide cardioprotection by suppressing complex I activity (Di Lisa and Bernardi 2015). NO can inhibit complex I activity via S-nitrosylation (up to 40% reduction) (Burwell et al. 2006) and has been reported to cause a reversible conformational change in the enzyme (Vicente et al. 2016). However, NO derivatives can also inhibit other components of the mitochondrial respiratory chain: at 1  $\mu$ M, NO inhibits complex III reversibly; in addition, S-nitrosylating reagents such as S-nitrosothiols (SNOs) or  $N_2O_3$  can inactivate complex I, and peroxynitrite through oxidation can inhibit most mitochondrial proteins irreversibly (Cooper and Giulivi 2007). Unlike NO, there is no clear evidence that either CO or  $H_2S$  can directly modulate complex I activity.

Complex II (succinate dehydrogenase) has been linked to the putative mitochondrial ATP-sensitive  $K^+$  channel ( $mK_{ATP}$ ), an important component in cardioprotection by ischemic preconditioning (Di Lisa and Bernardi 2015). Atpenin A5 (AA5), a specific complex II inhibitor, opens the  $mK_{ATP}$  channel at a concentration below the complex II-inhibiting dose (Burwell et al. 2009). As mentioned in section 4, CO,  $H_2S$ , and NO can activate the  $K_{ATP}$  channel in the plasma membrane (Fig. 2 and Table 2). Therefore, the  $mK_{ATP}$  channel may also be a target of all three gases, although direct evidence for this is lacking. In particular, the nitroxyl (HNO) donor Angeli's salt is acknowledged for its cardioprotective effect, and its potential human therapeutic role is under investigation (Burwell et al. 2009; Bianco et al. 2017).

Complex III (cytochrome bc1 complex or ubiquinol: cytochrome c oxidoreductase) is where electrons and dehydrogenases entering the Q cycle from the upstream complexes I and II converge. Complex III is vital for the movement of electrons from ubiquinol to cytochrome c and is also where ROS is generated within mitochondria. Unlike with complexes I and II, the electron supply cannot proceed in the absence of complex III, and blocking complex III leads to complete inhibition of the respiratory chain (Burwell et al. 2009). Complex III inhibitor antimycin A-mediated protection against I/R injury, which is abrogated by antioxidants, poses a possible ROS-related role (Vicente et al. 2016). In contrast, the complex III inhibitor myxothiazol suppresses ROS production by complex III, but does not confer a cardioprotective effect (Burwell et al. 2009). In complex III, cytochrome c adopts a hexa-coordinate arrangement, such that CO,  $H_2O_2$ , NO, and  $O_2$ , cannot approach the heme moiety in cytochrome c. Loss of the axial ligand results in a penta-coordinate heme, allowing cytochrome c to initiate peroxidase activity (Belikova et al. 2006). NO, released from S-nitrosoglutathione (GSNO) or spermine-NO complexes or generated by mtNOS, inhibits complex III electron transfer in an oxygen concentration-independent manner. This inhibition of complex III by NO reduces I/R injury via ROS production, similar to an antimycin A (Iglesias et al. 2015). Via nitrosylation, NO can inhibit the peroxidase activity of the cardiolipin/cytochrome c complex, thereby protecting mitochondria from outer membrane permeabilization (Burwell and Brookes 2008).

Complex IV (cytochrome c oxidase) constitutes the final rate-limiting electron acceptor of the mitochondrial respiratory chain and contains a heme a3-copper B (CuB) binuclear center, to which oxygen binds during their respective reduced states

(i.e.,  $\text{Fe}^{2+}$  and  $\text{Cu}^+$ ) (Pun et al. 2010). In heart cells, complex IV is a major  $\text{O}_2$ -consumption site; therefore, enzyme activity modulation is significant for cardiomyocyte oxygenation. Fully assembled complex IV co-localize with complexes I and III, forming larger assemblies termed super-complexes (Acin-Perez et al. 2008). When inhibited, respiratory chain electron flux (and therefore ATP synthesis) declines (Vicente et al. 2016; Cooper and Brown 2008). Substantial complex IV dysfunction causes an energy crisis owing to reduced ATP production, lactic acidosis, and increased mitochondrial ROS formation (Srinivasan and Avadhani 2012). In addition, electrons donated from complex III cannot be accepted by complex IV, resulting in increased ROS production owing to the leaked electrons. Direct complex IV activity inhibition occurs through inhibitors that interfere with the catalytic cycle, or when it binds at distal allosteric sites.

$\text{CO}$ ,  $\text{H}_2\text{S}$ , and  $\text{NO}$  can inhibit complex IV with differing kinetics (Vicente et al. 2016; Cooper and Brown 2008) and can confer cardioprotective effects against I/R injury through various mechanisms (Cebova et al. 2016; Vandiver and Snyder 2012; Burwell et al. 2009).  $\text{CO}$  serves as a reversible and competitive inhibitor, decreasing the  $K_M$  of complex IV for  $\text{O}_2$  by binding at the  $\text{O}_2$ -reactive, fully reduced heme  $\text{a}_3$ - $\text{CuB}$  active site (Vicente et al. 2016; Petersen 1977; Boczkowski et al. 2006). The cardioprotective effect of  $\text{CO}$  is largely due to its activity at complex III, because when complex III is inhibited, the effect of  $\text{CO}$  is lost.  $\text{H}_2\text{S}$  has been described as a noncompetitive inhibitor of isolated mitochondrial complex IV (Owens 2010); for example,  $\text{H}_2\text{S}$  affects the  $V_{\max}$  of complex IV, but not the  $K_M$  for  $\text{O}_2$ . Under normal conditions,  $\text{H}_2\text{S}$  can serve as a mitochondrial respiratory chain substrate (Fig. 3). A low concentration of  $\text{H}_2\text{S}$  stimulates  $\text{O}_2$  consumption and increases the membrane potential, whereas at high concentrations ( $>20 \mu\text{M}$ ) it inhibits  $\text{O}_2$  consumption at cytochrome oxidase (Vicente et al. 2016). Complex IV inhibition by  $\text{H}_2\text{S}$  appears to be reversible and independent of the oxygen concentration (Vicente et al. 2016). This inhibition of complex IV by  $\text{H}_2\text{S}$  is pH-dependent, as the  $K_i$  value of complex IV drops significantly at an acidic pH. Considering the low cellular pH during ischemia, a small portion of  $\text{H}_2\text{S}$  can more efficiently inhibit complex IV under these conditions. By competing with  $\text{O}_2$  at complex IV,  $\text{NO}$  slows the electron flux, reduces  $\text{O}_2$  consumption, and is catabolized to the nitrite ion (Pearce et al. 2002). The complex IV-inhibition pathway used by  $\text{NO}$  varies depending on the electron flux and  $\text{O}_2$  levels. As mentioned above,  $\text{NO}$ -dependent effects such as S-nitrosylation at complex I and III may further decrease electron flux along the ETC (Iglesias et al. 2015; Dahm et al. 2006). In contrast to  $\text{CO}$  and  $\text{H}_2\text{S}$ ,  $\text{NO}$  reversibly inhibits complex I-, II-, and IV-dependent respiration (Cassina and Radi 1996). It has been suggested that this inhibitory effect of  $\text{NO}$  is first initiated at complex IV, which consequently leads to a secondary electron transport impairment and, hence, impaired glutamate-, malate-, and succinate-driven respiration (Heales et al. 1999). The lack of an immediate effect of the peroxynitrite anion ( $\text{ONOO}^-$ ), an  $\text{NO}$  intermediate, on complex IV might imply that intracellular, time-dependent mechanisms are required to disrupt this complex. It should also be mentioned that  $\text{CN}$  may serve as another kind of gasotransmitter involved in cardioprotection (Lee and Han 2017).  $\text{CN}$  binds to the binuclear center of oxidized heme  $\text{a}_3$  in complex IV, in a non-competitive

manner with respect to  $O_2$ , and thus suppresses complex IV activity (Petersen 1977). Endogenous enzymes involved in the synthesis of CN are less known. It is possible that CN can be produced endogenously and function as a gasotransmitter, as shown in a study of rat brain tissues where opiate receptors increased and muscarinic receptors decreased cyanide production via G protein-independent mechanisms (Gunasekar et al. 2004). It has been hypothesized that complex IV activity will be more rate limiting for respiration depending on complex IV partial inhibition in mitochondria by one gas, resulting in an increased sensitivity toward other gases (Cooper and Giulivi 2007). For example, when  $H_2S$  is rapidly expended as a respiratory chain substrate, complex IV inhibition by any gas (or hypoxia) reduces  $H_2S$  expenditure, thus increasing available  $H_2S$  for oxidase inhibition. In addition, CO surplus can replace NO, and  $H_2S$  surplus can replace HCN. However, further studies need to be performed to determine whether a combination of these gases can efficiently inhibit complex IV.

The final step of oxidative phosphorylation involves mitochondrial complex V ( $F_1F_0$ -ATP synthase). This approximately 600-kDa membrane-bound protein complex is composed of the rotary catalytic motor  $F_1$  and the membrane turbine (ion pump) motor  $F_0$ , linked by central and peripheral stalks (Long et al. 2015; Yoshida et al. 2001). Proton translocation occurring in complexes I, III, and IV all play roles in complex V-mediated ATP production. The  $F_1F_0$ -ATP synthase is a two-faced enzyme complex responsible for ATP synthesis and hydrolysis in a manner dependent on oxygen-mediated electron flow. If the respiratory chain does not generate a proton gradient, the  $F_1F_0$ -ATP synthase couples proton pumping and ATP hydrolysis in order to sustain mitochondrial membrane potential (Di Lisa et al. 2007). Although  $H^+$  leaking wastes energy, it does not pose any adverse effect on the overall cellular ATP synthesis under a high workload because it only occurs at consequential amounts when ATP is not needed (i.e., when the [ADP] is low) (Brookes 2005). Mitochondrial  $H^+$  leaking and ROS production constitute a mechanistically linked odd couple, and mild uncoupling caused by a reversed mode of  $F_1F_0$ -ATP synthase has been shown to be a cardioprotective phenomenon found in ischemic preconditioning (Brookes 2005). Under pathophysiological conditions, wherein calcium concentrations increase in the mitochondrial matrix, the elevated nitrite concentrations in vivo may selectively inhibit the ATPase activity sustained by  $Ca^{2+}$ , without affecting that activated by  $Mg^{2+}$  (Nesci et al. 2016). Experimental data have suggested that S-nitrosylation of the  $F_1F_0$ -ATP synthase (mediated by NO) is protective, shielding the cysteine residues from oxidation, thereby protecting the proteins from irreversible oxidation at the start of reperfusion (Sun et al. 2007). Although it is unclear whether there is a direct connection between complex V inhibition and Cu/Zn-superoxide dismutase (SOD) activation, NaHS can activate Cu/Zn-SOD in a cell-free system without any other cellular components and, thus, may be involved in the indirect ROS-reducing activity of  $H_2S$  (Okubo et al. 2012).

## 5.2 Mitochondrial Bioenergetics and Biogenesis Associated with CO, H<sub>2</sub>S, and NO Action

The basic roles of CO, H<sub>2</sub>S, and NO involved in the control of mitochondrial metabolism will be considered separately. Apart from a cardioprotective role for CO, H<sub>2</sub>S, and NO during I/R, they can also participate in mitochondrial bioenergetics by directly acting as a substrate in oxidative phosphorylation (Bouillaud and Blachier 2011), or indirectly by regulating the activities of enzymes involved in glycolysis. CO upregulates metabolites in the re-methylation cycle and downregulates those in the trans-sulfuration pathway by inhibiting CBS (Hishiki et al. 2012). In addition, CO acutely causes decreased levels of GSH, which is associated with CBS inhibition (Hishiki et al. 2012). CO-mediated CBS inhibition causes a switch of glucose utilization towards the pentose phosphate pathway through the demethylation of 6-phosphofructo-2-kinase/fructose-2,6-bis-phosphatase 3 (PFKFB3) and eventually suppresses oxygen metabolism (Takano et al. 2010; Yamamoto et al. 2011). It has recently been shown that H<sub>2</sub>S exerts complex actions with respect to mitochondrial energetics. H<sub>2</sub>S is a substrate for the mitochondrial electron transport chain in mammals (Fig. 3), and in this regard, it is comparable to succinate (Bouillaud and Blachier 2011). At lower concentrations, when compared with NADH oxidation, sulfide oxidation is not highly efficient in terms of the energy yield. However, H<sub>2</sub>S has several advantages over NADH. First, it diffuses across membranes with ease. Second, high affinity of SQR for H<sub>2</sub>S (Fig. 1) ensures that H<sub>2</sub>S is a potent respiratory “fuel” at low, non-toxic levels (Vicente et al. 2016). H<sub>2</sub>S bioavailability inside cells is regulated by H<sub>2</sub>S-synthesizing and -consuming enzymes. Sulfide is considered as an “emergency” substrate at selected body regions and under specific physiological or stressful phenomena, based on its properties (Vicente et al. 2016). In addition, H<sub>2</sub>S can induce S-sulfhydration of F<sub>1</sub>F<sub>0</sub>-ATPase at C244 and C294, both in vitro and in cell-based assays, and this modification of F<sub>1</sub>F<sub>0</sub>-ATP synthase can increase its enzymatic activity (Modis et al. 2016). H<sub>2</sub>S, acting as a free radical scavenger, can neutralize mitochondrial reactive-nitrogen and reactive-oxygen species, thus preserving mitochondrial integrity (Mottetlini and Otterbein 2010). H<sub>2</sub>S inhibits the mitochondrial PDE2A isoform, which can then elevate mitochondrial cAMP levels. Because the mitochondrial membrane is not permeable to cAMP, the mitochondrial isoform PDE2A does not communicate with the cytosolic cAMP system; thus, it plays a vital role in regulating and fine-tuning the mitochondrial ETC by phosphorylating mitochondrial ETC proteins (Modis et al. 2013). In contrast, H<sub>2</sub>S is produced both by CSE and CBS, and stimulates angiogenesis, bioenergetics, and cell proliferation in colon cancer (Szabo et al. 2013).

NO can regulate mitochondrial bioenergetics through a variety of different mechanisms. With respect to glycolytic enzymes, NO can inhibit glyceraldehyde 3-phosphate dehydrogenase (GAPDH) through S-nitrosylation. GAPDH can also be inhibited by NO-derived nitro-lipids (nitro-oleate and nitro-linoleate) through reversible nitrosylation at a critical active site cysteine (Burwell et al. 2009). Unlike nitrosylation, the sulfhydration of GAPDH increases its catalytic activity by over 700% (Vandiver and Snyder 2012). GAPDH activation by sulfhydration is



physiologically important, as the total GAPDH activity of liver extracts is approximately 25–30% lower in mice lacking CSE, despite having normal levels of the GAPDH protein (Vandiver and Snyder 2012). Contrary to this finding, it was previously reported that GAPDH S-sulfuration mediated by  $\text{H}_2\text{S}$  and polysulfides inhibited GAPDH activity to approximately 42% of control (Jarosz et al. 2015). The Krebs cycle involves enzymes that are high in metal centers and reactive thiols, making them excellent targets for CO,  $\text{H}_2\text{S}$ , or NO regulation (Burwell and Brookes 2008). However, little evidence exists that CO,  $\text{H}_2\text{S}$ , and NO can affect these enzymes; thus, this area is open to future research.

In contrast to mitochondrial bioenergetics, which reflects the modulation of enzymatic activities in a given context, mitochondrial biogenesis refers to an increase in the mitochondrial DNA (mtDNA) content and mass (Diaz and Moraes 2008; Hom and Sheu 2009). A detailed description regarding mitochondrial biogenesis is beyond the scope of the current review. CO and NO can stimulate mitochondrial biogenesis in the rat myocardium (Kajimura et al. 2010; Valerio and Nisoli 2015; Nisoli et al. 2004; Suliman et al. 2007). CO has been ascribed a (pre)-conditioning role by stimulating mitochondrial ROS production and activating mitochondrial biogenesis (Zobi 2013; Piantadosi et al. 2008), as these actions of CO are blocked by the antioxidant system (Suliman et al. 2007; Almeida et al. 2015). CO can induce the mRNA and protein expression of NF-E2-related factor (Nrf)-1 (Suliman et al. 2007). CO-mediated mitochondrial biogenesis has been suggested to occur through stimulation of Nrf2 and/or the Akt signaling pathway, both of which are involved in mitochondrial biogenesis (Piantadosi et al. 2008). Because synthesized mitochondria in NO-induced mitochondrial biogenesis can fully produce ATP through OXPHOS, it was suggested that NO–cGMP-related pathways might be involved (Nisoli et al. 2004). The mtDNA content is dramatically reduced in CSE gene-knockout mice, and this reduction in the mtDNA content can be reversed by supplementation with  $\text{H}_2\text{S}$ , provided as NaHS (Li and Yang 2015). In addition,  $\text{H}_2\text{S}$  can stimulate mitochondrial biogenesis, as has been demonstrated in the liver (Untereiner et al. 2016), although there is less direct evidence that  $\text{H}_2\text{S}$  can regulate mitochondrial biogenesis in the heart. The mitochondrial transcription factor A (TFAM) functions as a master transcriptional activator for mtDNA replication (Clayton 1991).  $\text{H}_2\text{S}$  can participate in mtDNA replication and mitochondrial biogenesis by suppressing mitochondrial TFAM methylation, which suppresses TFAM transcription (Li and Yang 2015). Collectively, these findings indicate that impairment in the proper production of CO,  $\text{H}_2\text{S}$ , or NO, or their supply, may lead to a failure in both the fine-tuning of metabolism and mitochondrial contents, resulting in decreased mitochondrial mass and DNA content.

Collectively, CO,  $\text{H}_2\text{S}$ , and NO can cooperate in the inhibition of complex IV and confer cardioprotection by reversibly inhibiting the mitochondrial respiratory chain and regulating ROS production. In dys-synchronous heart failure, mitochondrial ATP synthase activity is significantly reduced owing to the oxidation of S–S bonds by GSSG (S-glutathionylation) on cysteine residues of either the  $\alpha$ - or  $\gamma$ -subunits of ATP synthase (Kondo et al. 2013). This functional impairment of ATPase shown in heart failure might inspire development of an intervention, based on the redox-regulating properties of CO,  $\text{H}_2\text{S}$ , and NO.

## 6 Possible Crosstalk Among CO, H<sub>2</sub>S, and NO

It is possible that direct chemical crosstalk occurs between H<sub>2</sub>S and NO, owing to their high reactivities. By reacting in cells or tissues, another new signal transducer may be formed that acts in either the same, or a different manner, compared to the parental molecules. For example, oxidized NO can be reduced in the presence of H<sub>2</sub>S leading to the formation of thionitrous acid (HSNO) as an intermediate, or the release of NO from GSNO. NO<sup>+</sup>, NO, and NO<sup>−</sup> can be produced through HSNO metabolism at the cellular level, with each product having distinct physiological consequences (Sen 2017). Furthermore, the reduction and direct displacement of HSNO by H<sub>2</sub>S (or by a direct product of the reaction between the NO donor sodium nitroprusside and H<sub>2</sub>S) results in the formation of yet another intermediate product, HNO, which can regulate protein functions through redox switches (Filipovic et al. 2013; Ge and Moss 2012). However, direct chemical crosstalk between CO, H<sub>2</sub>S, and NO, and the subsequent production of new secondary signaling molecules constitutes an emerging research field, and this aspect of crosstalk is not addressed further in this review.

Owing to the complex interconnectivity between CO, H<sub>2</sub>S, and NO, and the absence of exact detection methods, it is often difficult to determine the biological mechanisms associated with each isolated species. CO, H<sub>2</sub>S, and NO each promote mitochondrial signaling (Fig. 2) and ion channel activation (Fig. 3) in their own way. The cellular effects and signaling mechanisms of these gasotransmitters are spatiotemporal- and cell type-specific (Wang 2012); thus, the related signal-transduction pathways in cells could be intertwined and orchestrated in regulating vasodilation, promoting angiogenesis, attenuating apoptosis, and exerting antioxidant activities (Wang 2014; Polhemus and Lefer 2014). Apart from direct chemical crosstalk leading to the formation of a new signaling molecule, four possible crosstalk mechanisms exist between CO, H<sub>2</sub>S, and NO, namely: (1) competing for heme in heme-containing proteins, (2) regulating the production of other gases by regulating enzyme activities, (3) competing with post-translational modification sites in proteins (Kajimura et al. 2010; Rochette et al. 2013), and (4) forming hybrid molecules between two different gasotransmitters and functioning as a reactive intermediate or a biological mediator. However, the crosstalk that occurs between CO, H<sub>2</sub>S, and NO in the cardiovascular system is not limited to these four points.

### 6.1 *Competing and/or Synergizing at Heme-Containing Proteins*

Although differing in production and signaling pathways, the actions of CO, H<sub>2</sub>S, or NO can converge on the same protein targets. Crosstalk between CO, H<sub>2</sub>S, and NO may converge at the modulation of heme-containing proteins, as CO, H<sub>2</sub>S, and NO can bind to the heme-moiety in these proteins and modify their structure (Kajimura



et al. 2010; Rochette et al. 2013). One of the most extensively studied heme-dependent proteins is GC, which is responsible for generating the second messenger cGMP and plays a central signaling role in cardiovascular function and blood pressure regulation (Matterlini and Foresti 2017; Francis et al. 2010; Buys and Sips 2014). Particulate GC, which comprises the membrane-bound form of GC, is activated by natriuretic peptides (Garbers et al. 2006). sGC is a cytosolic histidine-ligated, heme-containing  $\alpha/\beta$  heterodimeric enzyme. Although  $O_2$  does not bind to sGC even in a 100%  $O_2$  atmosphere (Matterlini and Foresti 2017), CO and NO can bind to the heme moiety of sGC and thereby increase its enzymatic activity (Derbyshire and Marletta 2012). It is noteworthy that CO binds only ferrous ( $Fe^{2+}$ ) heme, whereas NO can bind both the  $Fe^{2+}$  and  $Fe^{3+}$  forms. Compared with NO, CO is a weak stimulator of sGC. CO can possibly serve as partial agonist to either facilitate or inhibit NO-mediated activation of sGC (Hartsfield 2002; Traylor and Sharma 1992). The capability of CO to “mimic” NO in activating sGC is a possible mechanism by which CO can affect NO signaling. Although no evidence has been shown that  $H_2S$  actually interacts with sGC (Wang 2012),  $H_2S$  can inhibit PDE-5, an enzyme that can degrade cGMP. Once built, targets of cGMP include cGMP-dependent protein kinases, ion-gated channels, and PDEs, which in turn can modulate physiological functions such as neurotransmission, platelet aggregation, and vasodilation (Munzel et al. 2003).

In endothelial NO synthase knockout mice,  $H_2S$  did not have any effect on angiogenesis and wound healing. Conversely, removal of  $H_2S$  production through CSE gene silencing abolished NO-stimulated angiogenesis (Hosoki et al. 1997; Coletta et al. 2012a), because both  $H_2S$  and NO synergize to produce a net increase in cGMP that results in activation of PKG as well as vasodilator-stimulated phosphoprotein, its downstream effector (Coletta et al. 2012a). Reaction between  $H_2S$  and NO yields HNO (Whiteman et al. 2006) and/or polysulfides  $H_2S_n$ , which play important physiological roles and provide therapeutic targets for diseases involving these molecules (Miyamoto et al. 2017).

Notably, owing to sGC deactivation, NO-induced relaxation of smooth muscle cells ceases within a matter of seconds upon removal of free NO (Palmer et al. 1987). Upon consistent exposure to NO or NO donors (such as GTN), the maximal sGC activation decreases as a result of sGC desensitization (Derbyshire and Marletta 2012). Mechanistically, nitrate desensitization may occur by matriculation, which is the oxidative addition of NO to a thiol of both the  $\alpha 1$  (C243) and  $\beta 1$  (C122) subunits of sGC (Sayed et al. 2007), resulting in sGC inhibition (Derbyshire and Marletta 2012). When ATP and GTP are present (such as in physiological phenomena), the ferrous-nitrosyl species has low activity and is very stable at low NO concentrations (Cary et al. 2005). Increased NO concentrations fully, albeit transiently, activate sGC. When ATP levels suddenly decrease (for example, during cellular stress), the binding of GTP and NO to the heme of sGC is sufficient for full enzyme activity, albeit transiently as well. Both NO and CO can increase the activity of native  $BK_{Ca}$  channels in rat VSMCs; this same type of interaction with CO and NO has also been proposed for sGC regulation (Traylor and Sharma 1992). The excitatory effect of NO on  $BK_{Ca}$  channels is independent of the actions of CO because the  $\alpha$  subunit of

BK<sub>Ca</sub> is essential for the activation by CO, whereas the  $\beta$  subunit of BK<sub>Ca</sub> is necessary for NO action (Wu et al. 2002). When NO first occupies the  $\beta$  subunit of the BK<sub>Ca</sub> channel, NO can neutralize CO-mediated BK<sub>Ca</sub> channel excitation owing to its allosteric effect on the  $\alpha$  subunit of BK<sub>Ca</sub> (Wu et al. 2002). Thus, the continual presence of CO in VSMCs exerts a small effect on the NO sensitivity of BK<sub>Ca</sub> channels, so that vascular contractility can be acutely and precisely regulated by NO surges.

## 6.2 *Regulating the Production of Other Gas via Regulating Enzyme Activities*

Gasotransmitter-producing enzymes such as NOSs, HO-1, and CBS utilize specific substrates and cofactors. However, interactions among gas ligands do occur through heme moieties, thus affecting the enzymatic activity of each gas-producing enzyme. It has been suggested that NO and CO regulatory effects are connected; for example, a dysfunctional HO–CO system causes vascular resistance in the heart and lungs, resulting in systemic and pulmonary hypertension (Boczkowski et al. 2006). NO has a small effect on the production of CO and H<sub>2</sub>S, whereas CO appears to serve as an effective repressor of both NO and H<sub>2</sub>S production (Wesseling et al. 2015). CO can be differentially involved in the production of NO. CO is thought to inhibit NOS activity by coordination with the NOS prosthetic heme group (Fig. 1), although this inhibitory action has only been observed at extremely high concentrations ( $\approx 1$  mM). At relatively low concentrations, NO release from proteins can be prompted by CO, as can peroxynitrite production (Thorup et al. 1999). Conversely, NO can affect CO production by modulating cGMP-independent transcriptional upregulation of the gene for HO-1 and/or by inhibiting the decay of its mRNA (Hartsfield et al. 1997). In contrast, NO can bind to the heme moiety of the heme–HO-2 complex and thereby suppress HO-2 activity, which is dependent on the heme moiety for its enzymatic activity (Ding et al. 1999).

H<sub>2</sub>S is a potent regulator of the cellular redox status that controls oxidative stress, thereby conserving eNOS function and promoting NO production. CO can inactivate CBS through binding to Fe<sup>2+</sup>–CBS (Puranik et al. 2006), resulting in the decline of H<sub>2</sub>S production. CO-mediated inhibition of CBS likely triggers methylation or demethylation of different protein targets for varied durations owing to changes in the methionine and S-adenosylmethionine levels, which are involved in re-methylation cycling (Yamamoto et al. 2011). NO can bind to ferrous CBS more tightly (with a  $K_d < 0.23$   $\mu$ M) than CO. CBS activity is suppressed after CO or NO molecules bind to their respective cysteine or histidine sites in CBS (Vicente et al. 2014). In addition, the enzymatic activity of CSE is suppressed after NO binding (Asimakopoulou et al. 2013). In contrast, CSE-mediated H<sub>2</sub>S production can be augmented by an NO donor through elevated enzymatic activity and/or CSE induction in VSMCs (Zhao et al. 2001). Although tissue-specific regulation of H<sub>2</sub>S

production or the non-enzymatic release of  $\text{H}_2\text{S}$  moieties from cellular macromolecules caused by NO is possible, the reason why  $\text{H}_2\text{S}$  production was actually increased by NO in this experimental setting is puzzling. If the activity of CBS is suppressed, then the levels of  $\text{H}_2\text{S}$  will decrease (Vicente et al. 2014, 2016; Vandiver and Snyder 2012), whereas the levels of its substrate homocysteine, which is considered an independent risk factor for cardiovascular and thrombotic diseases (Fowler 2005), will increase (Vicente et al. 2014). In addition, reduced  $\text{H}_2\text{S}$  production can cause profound oxidative stress, decreased  $\text{BH}_4$  levels, and dysfunctional eNOS (such as with eNOS uncoupling) (Ertuna et al. 2017); thus, reduced NO levels further exacerbate the cardiovascular pathology (Polhemus and Lefer 2014). Experimentally, it has been found that the adverse effects of increased NO in an endotoxemia model were suppressed by supplying an exogenous  $\text{H}_2\text{S}$  donor (Vicente et al. 2016). It has also been found that the vascular  $\text{H}_2\text{S}$  synthesis/ $\text{H}_2\text{S}$  pathway was impaired in an L-NAME-induced hypertensive rat model (Zhao et al. 2003) and in eNOS $^{-/-}$  mouse carotid arteries (Ertuna et al. 2017). This condition of hypertension was not ameliorated by the CO/HO-1 system (Ulker et al. 2017), but was countered by the supply of exogenous  $\text{H}_2\text{S}$  (Cacanyiova et al. 2016a) or its substrate L-cysteine (Ertuna et al. 2017), indicating that  $\text{H}_2\text{S}$  can act as an alternative means to maintain vascular patency.  $\text{H}_2\text{S}$  causes dual modulation of the vascular tension (vasoconstriction and vasorelaxation), depending on its concentration. It has been suggested that  $\text{H}_2\text{S}$  can inhibit eNOS activity (Kubo et al. 2007) and/or quench NO, resulting in the conversion of NO to a nitrosothiol-like compound (Whiteman et al. 2006). NO production was also influenced by  $\text{H}_2\text{S}$  through the NO-dependent suppression of nNOS and iNOS activity (Heine et al. 2015). In contrast,  $\text{H}_2\text{S}$  can increase NO production by calcium-mediated eNOS phosphorylation independently of the PI3K–Akt pathway (Kida et al. 2013), cause Akt-dependent eNOS activation (Coletta et al. 2012b), or stabilize eNOS activity (Li et al. 2017) in endothelial cells.

### **6.3 Gasotransmitter Competition in the Post-translational Modification of Proteins**

During the post-translation modification of proteins, S-nitrosylation often impedes the functions of its targets, whereas sulfhydrylation enhances their activities (Mustafa et al. 2009b; Iciek et al. 2015). NO and  $\text{H}_2\text{S}$  can compete for site recognition at cysteine residues during nitrosylation and sulfhydrylation, respectively. For example, GAPDH sulfhydrylation leads to PSD95 degradation via Siah, whereas GAPDH nitrosylation results in cell death by activating the p300–CBP and p53 signaling axis. Another example of such regulation involves p65 (NF- $\kappa$ B). Sulfhydrylation of p65 allows it to interact with RPS3 and facilitates p65 transcriptional activation. In contrast, p65 nitrosylation prevents its transcriptional activation (Sen 2017). Occasionally,  $\text{H}_2\text{S}$  and NO are both required for certain physiological actions, such as vasorelaxation and angiogenesis (Mistry and Brewer 2017; Ishigami et al. 2009;

Coletta et al. 2012a). Protein tyrosine phosphatases (PTPs) are involved in numerous signal-transduction pathways (Tonks 2013). NO (or peroxynitrite)-mediated PTP inhibition can occur through either cysteine S-nitrosylation and dimerization or H<sub>2</sub>S-mediated inhibition through cysteine sulfhydration (Tonks 2013; Heneberg 2014). Importantly, NO- or H<sub>2</sub>S-mediated PTP1B inactivation is reversible by >90%, and the S-nitrosylation of PTP1B protects it against irreversible inactivation by ROS (Chen et al. 2008). Findings with rodent models have shown that eNOS inhibition causes peripheral insulin resistance, hypertension, and hyperlipidemia, and that NO donors can mimic the activity of insulin by stimulating glucose transport and metabolism in rodent muscles (Hsu and Meng 2010). This aspect of NO may be related to its ability to inactivate PTP1B, as well as related PTPs that are involved in enhancing insulin responsiveness in tissues targeted by insulin. PTEN is a phosphatase that negatively regulates phosphatidylinositol triphosphate levels and contains several cysteine residues that play key roles in the catalytic mechanism, as well as at allosteric sites. Pre-existing posttranslational modification of a protein may confer resistance or sensitivity to subsequent modification induced by a different pathway. For example, PTEN can be oxidized by NO (i.e., S-nitrosylation) and H<sub>2</sub>O<sub>2</sub> (i.e., disulfide bond formation), with these modifications leading to the suppression of PTEN activity and increased activity of the PI3K–Akt pathway. Endogenous H<sub>2</sub>S, not provided by NaHS, is involved in regulating the basal activity of PTEN via S-sulfhydration (Traylor and Sharma 1992). This activity of H<sub>2</sub>S is associated with persulfides (CysSSH groups) (Yuan et al. 2016) and polysulfides (H<sub>2</sub>S<sub>n</sub>), which are often considered mediators of in vivo H<sub>2</sub>S signaling (Greiner et al. 2013), providing resistance to oxidative attack by NO to inhibit PTEN activity (Ohno et al. 2015).

#### **6.4 Formation of Hybrid Molecules Acting as Intermediates or Signaling Mediators**

Few reports are available regarding the CO-mediated formation of intermediates from H<sub>2</sub>S and NO, owing to the chemically inert state of CO. Chemical interactions between H<sub>2</sub>S and NO can form intermediates and reaction products, such as HNO, S-nitrothiol (SNO<sup>−</sup>), H(S)S<sup>−</sup>, H(S)S<sup>−</sup>, and nitrosopersulfide (SSNO<sup>−</sup>), through various enzymatic and non-enzymatic reactions (reviewed in Cortese-Krott et al. 2015). Specifically, SSNO<sup>−</sup> is a perthionitrite (as opposed to a polysulfide) and can generate both NO and polysulfides at pH 7.4, acting as a dual-purpose signaling molecule with both sulfane sulfur and NO bioactivity (Cortese-Krott et al. 2015). The biological roles of these intermediate products and reaction products can differ from their parental molecules by eliciting distinct signaling responses (reviewed in Bianco et al. 2017; Kevil and Patel 2010; Wedmann et al. 2017). Currently, hybrid molecules produced from NO and H<sub>2</sub>S are being extensively investigated to elucidate their precise biological importance.

## **6.5 *Sensing of Oxygen and Transcriptional Regulation via Gas-Responsive Transcription Factors***

O<sub>2</sub> tension varies between tissues. CO, H<sub>2</sub>S, and NO are involved in complex crosstalk with O<sub>2</sub> that regulates red blood cell levels and the blood vessel tone, both playing important roles in O<sub>2</sub> delivery. The production of CO and NO can be somewhat dependent on cellular O<sub>2</sub> levels (Kolluru et al. 2016), as molecular O<sub>2</sub> is requisite for the enzymatic activities of HO-2 and nNOS. Thus, the production of CO by HO-2, and NO by nNOS, can be suppressed under hypoxic conditions that also regulate the steady-state expression of NOS enzymes at the mRNA and protein levels (Ho et al. 2012). Under hypoxic conditions, CO generation from HO-2 is reduced, leading to decreased CBS inhibition by CO and elevated H<sub>2</sub>S levels, which in turn contribute to sensory excitation in the carotid body (Prabhakar and Semenza 2012). Under normoxic conditions, however, O<sub>2</sub>-dependent CO generation from HO-2 inhibits CSE activity, leading to decreased H<sub>2</sub>S levels and lower sensory activity in the carotid body (Prabhakar and Semenza 2012). Unlike CBS, CSE found in the carotid body does not contain heme; thus, there is a need to define the mechanisms by which CO inhibits H<sub>2</sub>S generation in further studies (Prabhakar and Semenza 2012).

It has been suggested that gasotransmitters can modulate gene transcription by interacting with transcription factors containing heme as a prosthetic group. For example, CO can activate neuronal PAS domain protein 2 (NPAS2), which is an obligate dimeric partner of BMAL1 and is involved in regulating the circadian rhythm (Dioum et al. 2002). This result suggests a reciprocal relationship between heme biosynthesis and its degradation. H<sub>2</sub>S can block the induction of Brg1 expression, which is the central catalytic subunit of SWI-SNF, an ATP-dependent chromatin remodeling complex, although the associated mechanism remains unidentified (Alexander et al. 2013). Inhibition of VSMC proliferation caused by H<sub>2</sub>S has been shown to be closely related to Brg1-induced chromatin remodeling.

## **7 *Clinical Relevance of Gasotransmitters and Their Donors in the CVS***

Each of the three gases CO, H<sub>2</sub>S, and NO acts as vasodilators, in addition to being considered as broad-spectrum anti-inflammatory and cytoprotective agents (Cebova et al. 2016; Papapetropoulos et al. 2015a, b; Rochette et al. 2013; Beltowski 2015). Ischemic heart disease is a main cause of morbidity and mortality; thus, cardioprotective strategies focused on reducing the infarct size, the incidence of arrhythmias, and cardiac dysfunctions after ischemic insults have been extensively studied (Rossello and Yellon 2016; Ferreira 2010). Unfortunately, no medicine has been developed to treat ischemic heart disease clinically (Rossello and Yellon 2016). Similar to the previously identified cardioprotective roles of NO (Cohen et al. 2006;

Pagliari et al. 2001), both CO and H<sub>2</sub>S (in either endogenous or exogenous forms) have been extensively evaluated as novel cardioprotectants, and their numerous beneficial roles against I/R injury have been suggested based on experimental models (Andreaddou et al. 2015). However, demonstrating a direct cause-and-effect relationship between gases, their clinical effects, and the relevant underlying mechanisms is complicated (Kajimura et al. 2010; Calvert and Lefer 2010) and thus, in many cases, the long-term consequences and clinical benefits of these treatments remain to be determined (Milkiewicz et al. 2006). The most well-characterized roles of these gaseous molecules in vasorelaxation are closely associated with the sGC–cGMP–PKG pathway (Fig. 2), the modulation of ion channel activities (Table 2), or both. Although it has been suggested that excessive ROS production in the heart and blood vessels constitutes a pivotal etiological inducer of hypertension, or other cardiovascular diseases, clinical trials using different antioxidants (e.g., ascorbic acid, vitamin E, or  $\beta$ -carotene) have yielded contradictory outcomes (Pechanova and Simko 2009; Villanueva and Kross 2012). Moreover, the therapeutic efficacy of conventional drugs in managing metabolic syndromes is largely influenced by the concentrations or the supplied condition of CO, H<sub>2</sub>S, and NO (Pechanova et al. 2015). It is hoped that the translational science of these gasotransmitters involving endogenous signaling, acting either through concerted or separate modes of action, will provide new biological concepts for treating cardiovascular disease and aid in developing a new generation of drugs.

### ***7.1 Pathological Changes Accompanied by Impaired Endogenous Production of Gasotransmitters***

Abnormal CO metabolism and function may contribute to hypertension development and pathogenesis (Rochette et al. 2013; Ndisang et al. 2004). When the gene for HO-1 is knocked out, cardiac damage (following I/R injury) and secondary oxidative damage are both exacerbated, whereas cardiac HO-1 overexpression following I/R injury reduces the infarction size as well as other markers of damage (Yet et al. 2001). CO production after HO-1 induction in vascular tissue and its subsequent activation of cGMP signaling decreased in spontaneously hypertensive rats and other models of hypertension (Cacanyiova et al. 2016a). Disturbances in H<sub>2</sub>S homeostasis may also promote hypertension (Sikora et al. 2014). In various experimental models, spontaneous hypertension (Du et al. 2003), along with high-salt-induced hypertension in Dahl rats (Huang et al. 2015), is closely associated with decreased plasma levels of H<sub>2</sub>S, and the hypertensive condition can be alleviated by supplementation with hydrogen sulfide (e.g., NaHS). In angiotensin (AT) II-infused mice, NaHS ameliorated hypertension as well (Al-Magableh et al. 2015). CSE<sup>−/−</sup> mice appear normal, but exhibit age-related hypertension and reduced endothelium-dependent vasodilation (Yang et al. 2008), indicating that CSE-derived H<sub>2</sub>S is an important mediator of vascular reactivity and blood pressure. However, in a different

study, CSE<sup>-/-</sup> mice on a low-cysteine diet were normotensive albeit highly vulnerable to acute lethal myopathy and oxidative injury (Ishii et al. 2010). The discrepancy in physiological outcomes shown in CSE<sup>-/-</sup> mice might be associated with different genetic backgrounds in these animals and the dietary composition, raising the possibility that the manifestation of conditions related to H<sub>2</sub>S insufficiency may vary with genetic and/or nutritional conditions. Unlike with H<sub>2</sub>S insufficiency, CSE-overexpressing transgenic mice showed a significant decrease in infarction compared to wild-type mice. In a pressure-overload heart-failure model, CSE<sup>-/-</sup> mice also exhibited exacerbated left ventricular dysfunction, whereas CSE-overexpressing transgenic mice showed suppression of aberrant cardiac structures and cardiac dysfunction, compared to wild-type mice (Polhemus and Lefer 2014). In view of atherosclerosis development, it has been suggested that decreased endogenous H<sub>2</sub>S production renders CSE<sup>-/-</sup> mice more susceptible to vascular remodeling and early-onset atherosclerosis (Mani et al. 2013).

Obesity and diabetes comprise metabolic disorders that are highly associated with cardiovascular complications. Data from several pharmacological studies, as well as transgenic models, suggest that cysteine and H<sub>2</sub>S participate in metabolic regulatory processes such as glucose homeostasis and/or insulin resistance (Carter and Morton 2016). Notably, obesity is associated with elevated plasma cysteine levels and reduced H<sub>2</sub>S levels, whereas diabetes is associated with reduced levels of cysteine and H<sub>2</sub>S (Jain et al. 2010; Whiteman et al. 2010). However, as reviewed in Carter and Morton (2016), several genetic models associated with altered sulfur metabolism did not match well with clinical data found when studying patients with obesity and diabetes. In diabetic nephropathy, H<sub>2</sub>S can preferentially inhibit renal inflammation and fibrosis, and favor hyperglycemic (high blood glucose) effects (Dugbartey 2016). Beyond impairment of the H<sub>2</sub>S-producing system, several pathophysiological conditions are associated with H<sub>2</sub>S overproduction, including sepsis. In addition, disturbances in H<sub>2</sub>S or NO signaling may affect the therapeutic efficacy of conventional drugs used to manage metabolic syndromes. Although the mechanisms of many anti-diabetic and anti-hyperlipidemic drugs are not directly related to NO signaling, some degree of interaction with NO-related pathways does occur (Pechanova et al. 2015). For example, anti-diabetic agents such as biguanides (e.g., metformin) and dipeptidyl peptidase-4 (DPP-4) inhibitors, but not sulfonylureas, may positively affect tissue NO availability associated with eNOS phosphorylation and NO signaling, thereby providing a promising tool for treating cardiac complications in patients with metabolic syndromes (Pechanova et al. 2015). Statins, being HMG-CoA reductase inhibitors improve cardiac NO metabolism, suggesting that biochemical differences in wide-ranging pathologies might significantly influence the beneficial pleiotropic effects of these drugs (Pechanova et al. 2015). Experimental evidence shows that administering cholesterol-reducing medications that stimulate the PPAR family of nuclear receptors leads to a restored balance in NO metabolism, which is disturbed by hyperlipidemia (Pechanova et al. 2015). In addition, long-term dietary nitrite/nitrate deficiency could cause endothelial dysfunction, cardiovascular death, and a metabolic syndrome-like condition in mice (Kina-Tanada et al. 2017).



## 7.2 Hypertension

Hypertension is distinguished by heightened vascular contractility, concomitant increases in oxidative stress, enhanced vascular inflammation, and vascular remodeling (Rochette et al. 2013). CO, H<sub>2</sub>S, and NO induce vasorelaxation, in either a separate or concerted manners, owing to their functional convergence in similar networks (Wang 2012). For example, as shown in Table 2 and Fig. 2, numerous ion channels involved in the relaxation of VSMCs are at least in part, immediate targets for CO, H<sub>2</sub>S, and NO actions. With regard to the vascular system, the vasodilatory effect of NO is largely dependent on its production in endothelial cells, whereas those of both CO and H<sub>2</sub>S are primarily initiated in smooth muscle cells. In a pulmonary arterial hypertension (PAH) model, inhaled CO both reversed pulmonary hypertension and promoted negative pulmonary vascular remodeling, albeit only when the eNOS–NO axis was functional (Zuckerbraun et al. 2006). CO can increase eNOS expression in pulmonary artery endothelial cells, whereas the beneficial effect of CO in PAH was suppressed in the absence of the eNOS–NO axis (Wang 2012). Endothelium injury and improper expression of NO-producing enzymes (NOSs), leading to impaired NO production, is associated with numerous cardiovascular conditions such as atherosclerosis, arterial thrombotic disorders, coronary heart disease, heart failure, hypertension, and stroke. Although vasorelaxatory activities of CO, H<sub>2</sub>S, and NO are commonly found, the opposite effect (vasoconstriction) can occur in some situations. As with chronic nitroglycerin (GTN) therapy, the cause of the rapid loss of hypotension has been identified; this nitrate-tolerance (or nitrate-resistance) phenomenon was excellently discussed in another review (see Daiber and Munzel 2015). Oxidative stress itself, as well as the suppression of GTN biotransformation, desensitization of downstream of NO-signaling pathways, epigenetic changes, and other mechanisms may contribute to pseudotolerance and true vascular tolerance (Daiber and Munzel 2015). Although a long journey remains ahead, owing to the limited clinical tools available for providing a steady supply of CO and H<sub>2</sub>S (as well as safety and clinical data), the possibility that both CO and H<sub>2</sub>S donors also mimic nitrate tolerance cannot be excluded.

The vasodilatory action of CO is closely associated with mechanisms involving sGC and the K<sub>Ca</sub> channel, and necessitates the neutralization of CO pro-oxidant activity (Bilban et al. 2008) as increased oxidant activity promotes the formation of isoprostanes that can constrict blood vessels (Rochette et al. 2013). Notably, biphasic actions of CO (vasodilation followed by vasoconstriction) on the newborn cerebrovascular circulation were found (Knecht et al. 2010). In this experiment, the cause of subsequent vasoconstriction might be connected to NOS inhibition after prolonged CO exposure, because L-arginine-mediated dilation diminished over time without the loss of dilation caused by the NO donor, sodium nitroprusside. Therefore, it has been suggested that episodic CO elevation constitutes an important factor for producing vasorelaxatory effects. However, this aspect of CO as shown in cerebrovascular regulation should be confirmed in the CVS.



Apart from the channel- and cGMP-PKG-regulatory roles of H<sub>2</sub>S, in endothelial cells, H<sub>2</sub>S can combine with the zinc ion in the active center of angiotensin-converting enzyme (ACE), which catalyzes the cleavage of AT I to AT II, and, thus, can directly inhibit ACE (Laggner et al. 2007). H<sub>2</sub>S causes vasorelaxation by suppressing AT II production from AT I (Laggner et al. 2007). In addition, AT II-mediated cardiac fibrosis and hypertrophy could be attenuated by supplying H<sub>2</sub>S (Snijder et al. 2015). Of interest, intra-cerebroventricular (ICV) infusion of NaHS in both spontaneous and AT II-induced hypertensive rat models caused a decrease in the mean arterial blood pressure and heart rate during ICV infusions (Sikora et al. 2014). Moreover, H<sub>2</sub>S secreted from periadventitial adipose tissue contributed blood pressure homeostasis (Fang et al. 2009). These remote effects of H<sub>2</sub>S on lowering the systemic blood pressure may provide a rationale for the local delivery of H<sub>2</sub>S.

During acetylcholine-mediated vasorelaxation, activated CaM (arising as a result of muscarinic acetylcholine receptor activation on endothelial cells) binds to and stimulates eNOS, CSE, and HO-2, resulting in the production of NO, H<sub>2</sub>S, and CO, respectively (Lundberg et al. 2015). At low concentrations (NaHS <100  $\mu$ M), however, H<sub>2</sub>S acts as a vasoconstrictor by inhibiting eNOS-derived NO production and the intracellular cAMP pathway. NO itself is a strong vasodilator, acting via the cGMP-PKG pathway, and stimulating K<sub>ATP</sub> channel opening to increase Ca<sup>2+</sup> reuptake by the endoplasmic reticulum, thereby decreasing intracellular Ca<sup>2+</sup> levels and reducing contraction. NO signaling is also stimulated by high concentrations of H<sub>2</sub>S, contributing to its vasodilatory effect. S-nitrothiols, derived from the interplay between NO and H<sub>2</sub>S, act as vasodilators mainly through NO signaling (Nagpure and Bian 2016; Modis et al. 2014).

### 7.3 Inflammation

The effects of CO, H<sub>2</sub>S, and NO on inflammation are complex and vary based on the concentration, delivery method, and disease model studied. There is no consensus regarding the anti-inflammatory roles of these gases in cardiovascular diseases, including atherosclerosis (ML et al. 2011; Lo Faro et al. 2014). Here, several examples of the anti-inflammatory roles of CO, H<sub>2</sub>S, and NO are briefly addressed (Tables 3, 4, and 5), although their exact underlying anti-inflammatory mechanisms also appear to be complex. The clinical utility of many drugs is limited owing to gastrointestinal complications including bleeding erosions. As for NO, previous data have shown that H<sub>2</sub>S and CO (or their chemical donors) can alleviate gastric ulcers by stimulating gastric blood flow (Magierowski et al. 2016); thus, their combined use for minimizing gastric damage from known drugs with adverse gastric complications is under development (Tables 3, 4, and 5).

CO can suppress cellular immune-effector functions by inhibiting the production of cytokines/chemokines, adhesion molecules, and hyper-proliferative signals (Woo et al. 1998; Otterbein et al. 2016); thus, CO may favor anti-inflammation (Ozen et al.

2015). Heme has been newly identified as another class of danger-associated molecular pattern (DAMP) molecules that can initiate a non-infectious immune response (Wegiel et al. 2015). As heme can stimulate HO-1 expression the heme content acts as an alarm for the subsequent recruitment of inflammatory cells that suppress inflammatory responses, such as a post-ischemic inflammation (Wegiel et al. 2015; Hinkel et al. 2015). The anti-inflammatory effects of H<sub>2</sub>S that are associated with inhibited lymphocyte and neutrophil infiltration to inflammatory sites has led to efforts to develop new therapeutic agents (Vandiver and Snyder 2012). Myeloperoxidase (MPO) is a heme-containing protein associated with numerous inflammatory events and cardiovascular diseases (Nussbaum et al. 2013). Recent findings suggest that endogenous sulfide levels possibly inhibit the activity of both circulating and endothelium-bound MPO in a reversible manner, and this is thought to be a major contributor to the activity of H<sub>2</sub>S during inflammation in the CVS (Papapetropoulos et al. 2015b). In addition, the underlying mechanism regarding the anti-inflammatory role of H<sub>2</sub>S may include its ability to scavenge peroxynitrite, a toxic NO derivative, in addition to other oxidants (Vandiver and Snyder 2012). H<sub>2</sub>S also sulphydrates the p65 subunit of NF- $\kappa$ B, promoting its attachment to co-activator ribosomal protein S3, thereby activating anti-inflammatory signals (Vandiver and Snyder 2012). Current attempts to develop new therapeutic agents involve coupling H<sub>2</sub>S donors with non-steroidal anti-inflammatory drugs (NSAIDs) (e.g., naproxen (ATB-346) and a diclofenac derivative) to overcome gastric irritation associated with NSAIDs (Vandiver and Snyder 2012). Beyond lowering blood pressure, NO exerts anti-inflammatory activity by blocking NF- $\kappa$ B binding to DNA via S-nitrosylation, followed by the stabilization of I $\kappa$ B, an endogenous NF- $\kappa$ B inhibitor (Marshall and Stamler 1999). NO can exert an anti-diabetic effect by prolonging insulin bioavailability, as NO can reversibly and non-competitively inactivate insulin-degrading enzymes via s-nitrosylation (Cordes et al. 2009). Based on the important physiological roles of NO, hybrid drugs with NO-releasing potential such as NCX-4016, NCX-6550, NCX-655 (Table 5), and others (Cordes et al. 2009) are aimed at maximizing drug efficacy and/or minimizing adverse effects caused by primary drugs, such as ACE inhibitors, AT2 receptor antagonists, and NSAIDs (Cordes et al. 2009).

## 7.4 Cardiac Hypertrophy

Cardiac hypertrophy, which is characterized by myocardial fibrosis, capillary rarefaction, inflammatory reactions, and cellular dysfunction, results in maladaptive ventricular remodeling and heart dysfunction (reviewed in refs. (Shimizu and Minamino 2016; Frey et al. 2004)). Patients with ischemic heart disease exhibited significantly elevated HO-1 immunoreactivity compared to those with congenital heart disease or dilated cardiomyopathy and myocarditis (Tang et al. 2010; Grabelius et al. 2002). Sustained HO-1 upregulation in the failing heart is a highly advantageous adaptation for counteracting detrimental left ventricular remodeling

via antioxidant, anti-hypertrophic, anti-fibrotic, and pro-angiogenic effects (Tang et al. 2010; Grabellus et al. 2002). Chronic upregulation of a compensatory system may not necessarily induce the same effects seen during acute stress response (for example, pro-inflammatory cytokines are acutely protective, but detrimental over the long term); hence, the short-term actions of HO-1 may not necessarily reflect a chronic setting (Tang et al. 2010). NO appears to suppress cardiac hypertrophy (Francis et al. 2010), as nNOS-knockout mice show age-related left ventricular hypertrophy, which is directly or indirectly associated with hypertension. Mutant mice without both nNOS and eNOS exhibited greater cardiac hypertrophy compared to mutant mice lacking only one of these enzymes (Bredt 2003). The additive effects on the cardiac phenotype of NO originating from different NOSs appear to emphasize the independent roles of nNOS and eNOS in cardiac function, suggesting a lack of cross-talk in cardiomyocyte signaling (Bredt 2003). H<sub>2</sub>S may also induce anti-hypertrophic remodeling through its antioxidant role, as transverse aortic constriction (TAC)-, or AT II-induced cardiac hypertrophy (both of which are highly associated with oxidative stress and reduced myocardial H<sub>2</sub>S levels), was significantly suppressed by exogenous supplementation of NaHS or sodium thiosulfate (Shao et al. 2017). An orally active H<sub>2</sub>S-releasing compound, SG-1002, can prevent cardiac enlargement, preserve left ventricular function, and reduce fibrosis after TAC in mice. However, this anti-hypertrophic effect of H<sub>2</sub>S was shown to be transitory because SG-1002 withdrawal led to the development of cardiac dilation and dysfunction (Kondo et al. 2013).

## 7.5 Cardiac Contractility

It will not be a simple task to decipher the effects of CO, H<sub>2</sub>S, and NO on cardiac contractility, as changes in cardiac functions reflect complex responses to systemic requirements and conditions. There is a strong possibility that the effects of CO, H<sub>2</sub>S and NO identified *in vitro* on cardiac contractility may be masked or repressed under normal conditions by whole-body regulatory interventions, such as neurological mechanisms (Dampney 1994). The effect of NO on cardiac contractility varies with its concentration and its compartmentation (Hare 2003; Ziolo et al. 2001). NO produced by eNOS acts primarily via a cGMP-dependent pathway, whereas NO produced by nNOS modifies RyRs in the sarcoplasmic reticulum through S-nitrosylation, which is acknowledged as a redox-dependent, cGMP-independent signaling element that mediates various NO activities. However, the regulatory roles of eNOS in the different aspects of cardiac contraction, relaxation, and heart rate are highly complex. Data from other studies have pointed to the role of iNOS in the pathogenesis of cardiac dysfunctions in mouse models of cytokine-induced cardiomyopathy (Serafim et al. 2012). NO exerts both acute effects, such as VSMC relaxation (Lefer and Lefer 1993), and chronic effects including the inhibition of VSMC proliferation and stimulation of endothelial cell regeneration (Guo et al. 1994). The negative inotropic effect of NO was first reported in isolated hamster

papillary muscles (Finkel et al. 1992). Conversely, in a later study involving the use of dissolved NO gas at 50 to 980 nM, the negative inotropic effect of NO was not found on isolated ventricular papillary muscles even though the coronary artery ring could be efficiently relaxed (Lefer and Murohara 1995). Endogenous NO production by eNOS has hardly any influence on the inotropic or lusitropic state of the heart under basal conditions, but has been observed to constantly oppose inotropic responses to  $\beta$ -adrenergic stimulation. Thus, NO is considered as a very weak regulator of the contractile function in unstimulated cardiac muscles.

H<sub>2</sub>S may reduce cardiac contractility by inhibiting LTCC activity (Table 2), as shown in isolated rat cardiomyocytes and papillary muscles (Sun et al. 2008) as well as K<sub>ATP</sub> channels (Geng et al. 2004b), or by blocking the cAMP–PKA pathway by inhibiting adenylyl cyclase activity (Yong et al. 2008). However, inconsistent changes in cardiac contractility mediated by H<sub>2</sub>S were found in experimental animals (Sun et al. 2008; Volpato et al. 2008a). For example, H<sub>2</sub>S inhalation at its metabolic-depressing concentration in mice revealed reversible and rapid decreases in heart rates and cardiac output, without changing the mean arterial pressure and stroke volume (Volpato et al. 2008a). In contrast, the cardiac contractility and heart rate were not significantly affected by the slow H<sub>2</sub>S-releasing compound GYY4137 (Li et al. 2008), but significant reductions in contractility and heart rate were caused by NaHS treatment in isolated rat hearts (Geng et al. 2004a). The different effects of NaHS and GYY4137 on cardiac contractility may, at least in part, rely on their mode of H<sub>2</sub>S release. Notably, pharmacological inhibitors of enzymatic H<sub>2</sub>S production have a less pronounced effect on cardiac and vascular contractility when compared to the administration of H<sub>2</sub>S donors in intact animals (Geng et al. 2004a; Zhao et al. 2001).

No equivocal results have been reported regarding hemodynamic responses to CO with respect to the species, CO dose level, CO dose rate, route of CO delivery, study duration, rest or exercise, state of consciousness, or anesthetic agent used (systemically reviewed in Penney 1988). For example, different reports have demonstrated no effect, a negative inotropic effect, or a positive inotropic effect of CO on cardiac contractility.

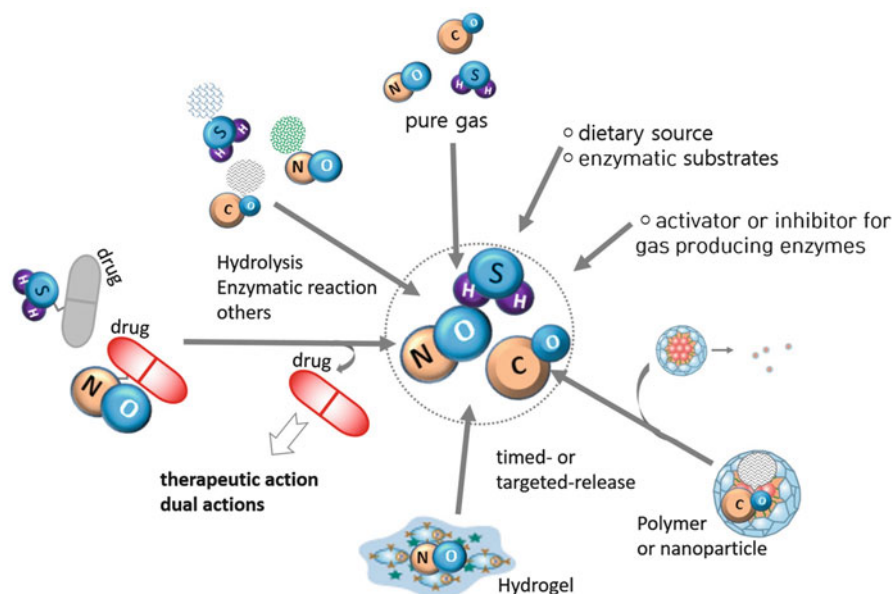
## 7.6 Other Roles of Gasotransmitters

A suspended animation-like state can occur in non-hibernating mammals as a conserved hypometabolic response to anoxia and/or environmental conditions, wherein all biological processes reversibly arrest, even movement and development (Blackstone et al. 2005). Hypothermic medical procedures for rapidly preserving an organism during ischemia have been under investigation to buy time for transport and resuscitative surgery (Liu et al. 2017). In addition to body temperature control, a suspended animation-like state can be promoted by the application of CO (Nystul and Roth 2004), H<sub>2</sub>S (Blackstone et al. 2005), and NO (Teodoro and O'Farrell 2003) in mice, *Caenorhabditis elegans*, or *Drosophila* embryos. It has been speculated that

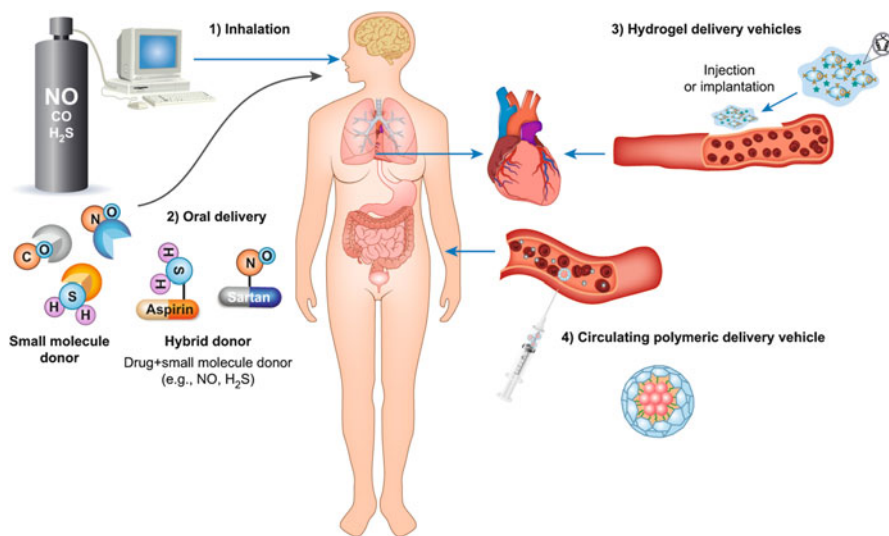
slowing respiration through complex I–V inhibition induced by CO, H<sub>2</sub>S, or NO can trigger suspended animation, owing to the resulting profound hypometabolism. However, the mere inhibition of complex IV activity does not appear to bring about suspended animation. In suspended animation associated with CO, H<sub>2</sub>S, or NO, some additional signals appear to be necessary for complete induction of the suspended phenomenon (Kajimura et al. 2010). It can be speculated that the regulated induction of a hypometabolic state contributes a medical benefit against tissue injury from I/R injury as described for mitochondria, pyrexia, and other traumas. CO and NO applications have also advanced in the field of organ transplantation and preservation (Lang et al. 2007). For example, CO suppressed the development of post-transplant arteriosclerotic lesions in rats receiving allogenic aorta transplants (Zobi 2013).

## 8 CO, H<sub>2</sub>S, and NO Donors and Their Delivery

Theoretically, the specific delivery of gaseous molecules at an appropriate therapeutic dose to a targeted tissue or organ, would be the best approach for using these molecules in clinical practice (Figs. 4 and 5). Given that CO, H<sub>2</sub>S, and NO play multiple biological roles, it is difficult to devise a specific form of these gaseous



**Fig. 4** Source of exogenous supplies of CO, H<sub>2</sub>S, and NO. CO carbon monoxide, H<sub>2</sub>S hydrogen sulfide, NO nitric oxide



**Fig. 5** Strategies for CO, H<sub>2</sub>S, or NO delivery. CO, H<sub>2</sub>S, or NO can be exogenously supplied by inhalation, via an oral route using a simple chemical simple donor (in combination with clinically used drugs), by injection with a form of hydrogel or circulating polymeric vehicle, or by implanting a hydrogel delivery vehicle. Substantial attention has been paid to issues of targeted gas delivery and the kinetics of exposure to ensure maximal effectiveness with minimal toxicity

molecules that would affect only one physiological function (Tables 3, 4, and 5). In addition, compared to traditional small molecules, gasotransmitters have short half-lives and greater likelihood for off-target effects, making targeted and localized delivery highly important for harnessing their therapeutic potential. Ideally, endogenous CO, H<sub>2</sub>S, or NO could be generated in cells proximal to specific targets because differences exist between endogenously and exogenously applied gasotransmitters (Mottetlini and Foresti 2017). Owing to the bell-shaped dose–response relationship that is commonly found, a specific therapeutic dose range and careful monitoring for toxicity indicators at higher levels is important for gasotransmitters (Szabo 2010). An additional factor that needs to be taken into consideration is that pre-existing levels of these agents will likely affect the overall biological responses to exogenously administered gasotransmitters (Szabo 2010). In gasotransmitter-based therapies, the maintenance of the therapeutic concentration for specific durations is also an important concern (Carpenter and Schoenfisch 2012). In this regard, four major factors that affect gasotransmitter concentrations over time, which should be considered, are (1) gas permeability across the membrane, (2) the physiochemical properties of the gasotransmitter, (3) characteristics of the local environment, and (4) the presence of scavenging systems and the chemical reactions that consume the gas. Gasotransmitters administered exogenously via their donors, by activation of enzymes involved in their production, or by releasing molecules entrapped in a nanostructure (e.g., a hard material or a hydrogel) should be able to interact with the same biological targets to produce the expected effects

(Kajimura et al. 2010; Cebova et al. 2016; Qian and Matson 2017; Beltowski 2015; Zobi 2013). In this section, the main methods for delivering CO, H<sub>2</sub>S, and NO are briefly addressed (Fig. 5); a more detailed description of all possible delivery methods (Qian and Matson 2017) is beyond the scope of this review.

Several gas-delivery methods (Figs. 4 and 5) are currently being developed pre-clinically, including inhalation, liquid formulation, or small-molecule gaso-transmitter donors that use either prodrugs that require conversion, or which spontaneously release the chemical donor (Qian and Matson 2017). Inhalation of a pure gas as CO, H<sub>2</sub>S, or NO obviates extraneous components related to gasotransmitter-releasing drugs; thus, inhalation therapy is favorable when targeting the lungs (Fig. 5). However, several concerns exist related to storage, administration, dose control, potential toxicity owing to reaction with endogenous ROS, the duration of action, and a lack of specific organ targeting (Qian and Matson 2017). Occasionally, inhalation of these gases can also produce systemic effects through the generation of oxidation products (e.g., nitrite and nitrate in the case of NO) or reaction products (e.g., S-nitrosothiols), which extend beyond local (intrapulmonary) actions (Dorsey et al. 2010).

Pro-drugs comprise bio-reversible drug derivatives that go through enzymatic and/or chemical changes in vivo in order to release the active parent drug, before being able to exert the desired pharmacological effect (Rautio et al. 2008; Ji et al. 2016). Pro-drug strategies have been demonstrated to be a very effective means of addressing delivery problems such as solubility, permeability, prolonged duration of drug action, and site-selective delivery (Rautio et al. 2008). Detailed descriptions of this aspect of drug development can be found in other excellent references (Kajimura et al. 2010; Szabo 2010; Qian and Matson 2017; Rochette et al. 2013; Beltowski 2015; Serafim et al. 2012; Rautio et al. 2008; Ji et al. 2016). Another strategy for delivering gasotransmitters is to integrate gasotransmitter-releasing functional groups into materials such as hydrogels, inorganic/organic hybrids, polymers, nanoparticles, and peptide-based materials to enable systemic administration. To elicit a tissue- or region-specific effect (Qian and Matson 2017), complex materials have been designed that release CO, H<sub>2</sub>S, or NO following injection or implantation at a specific delivery site (Qian and Matson 2017). These approaches all have potential clinical applications; however, advantages and disadvantages exist for each approach (Szabo 2010; Qian and Matson 2017).

Indirect approaches that can be used for therapy also exist, including the pharmacological stimulation of second-messenger pathways triggered by the gasotransmitter and supplementation with the substrate(s) or cofactors of the enzymes that produce the gaseous mediator (Szabo 2010).

## 8.1 CO Donors as Drug Targets for the CVS

CO acts as a vasodilator, as well as a broad-spectrum anti-inflammatory and cytoprotective agent (Qian and Matson 2017). Several types of CO-releasing



molecules (CORMs) are shown in Table 3. Inhaled CO has demonstrated a benefit in animal models with acute respiratory distress syndrome (ARDS), transplant rejection, circulatory shock, and vascular injury (Szabo 2010; Ghosh et al. 2010). There are many additional varieties of CORM beyond those containing boron or ruthenium centers, and not all CORMs contain metal centers. A great deal of concern exists over the toxicity of the ruthenium center of the most commonly studied CORMs (CORM-2 and CORM-3), and recent research suggests that manganese-based CORMs may be more suitable as they appear to be less toxic. CORMs can release CO from the metal core via a spontaneous reaction, thermal activation, hydrolysis in biological buffers, or in response to an external stimulus such as light (Zobi 2013; Bilban et al. 2008). The most frequent problems encountered in designing CORMs include issues with water solubility, biocompatibility, aerobic stability, stability in aqueous aerobic media, and issues beyond the basic properties of drug-like substances including absorption, distribution, metabolism, excretion, and toxicity. Similar to NO and H<sub>2</sub>S, CORMs work as systemic prodrugs as these are not targeted to specific receptors or macromolecules (Zobi 2013; Bilban et al. 2008). For the next generation of CORMs, tissue-specific delivery with controlled CO release is warranted (Kajimura et al. 2010). An important potential field for CORM application is organ transplantation. Currently, the use of cold storage procedures can curb ischemic injury and graft dysfunction in patients receiving transplants, although such procedures cannot completely prevent these effects. The use of CO as an organ preservative is currently being studied clinically. CO, either as a gas (saturated solution) or in the form of CORMs, can act as a protective adjuvant in preservation solutions (Rochette et al. 2013). In addition, methylene chloride, also known as dichloromethane, can be converted to CO in cells by carbonic anhydrase (Andersen et al. 1994). Thus, the use of methylene chloride is being examined in pre-clinical animal models of organ failure and transplantation (Wu and Wang 2005; Dorsey et al. 2010). Although the medical applications of CORMs appear to be promising, unanswered questions such as the site of action of CO and the sites of toxicity remain to be answered (Zobi 2013).

## 8.2 H<sub>2</sub>S Donors as Drug Targets for the CVS

As depicted in Table 4, currently obtainable H<sub>2</sub>S donors comprise inorganic sulfide salts (e.g., NaHS), synthetic organic slow-releasing H<sub>2</sub>S donors, H<sub>2</sub>S-releasing NSAIDs, cysteine analogs, nucleoside phosphorothioates, and plant-derived polysulfides (found in garlic) (Beltowski 2015; Vandiver and Snyder 2012; Dorsey et al. 2010). The curative effect of cholesterol-lowering statin drugs, at least in part, can be ascribed to the activation and increased expression of the endothelial isoform of NO synthase (Jacobson 2009). Statins may increase H<sub>2</sub>S synthesis by up-regulating CSE (Avanzato et al. 2014) and/or reducing its degradation by decreasing the coenzyme Q concentration (Beltowski 2015). In preclinical studies, inhaled H<sub>2</sub>S was advantageous in treating acute hemorrhagic shock, acute hypoxia,



and ventilator-induced lung injury (Szabo 2010). As an endogenous booster of H<sub>2</sub>S synthesis, the administration of cysteine or its derivatives (as H<sub>2</sub>S precursors) will need to be examined in vivo. Notably, parenteral administration of cysteine in rodents has been shown to have greater therapeutic effects (cardiac protection and vascular responses) comparable to those of H<sub>2</sub>S (Dorsey et al. 2010). Positive effects of H<sub>2</sub>S donors have been found when treating cardiovascular diseases including arterial hypertension, atherosclerosis, myocardial hypertrophy, myocardial infarction, and heart failure (Bilban et al. 2008; Kashfi and Olson 2013). H<sub>2</sub>S donors have also been shown in venules to prevent platelet aggregation and thrombus formation (Bilban et al. 2008). There are currently at least two cardiovascular H<sub>2</sub>S trials registered on [clinicaltrials.gov](http://clinicaltrials.gov) (Polhemus and Lefer 2014).

### 8.3 NO Donors as Drug Targets for the CVS

Some examples of NO donors are briefly outlined in Table 5. Each possesses features that can determine the rate of NO release, peak NO concentration, and NO-related species produced for a particular application (Cebova et al. 2016; Serafim et al. 2012; Oliver et al. 2010; Wang et al. 2002). Inhaled NO has shown beneficial effects in animal models of ARDS, circulatory shock heart failure, pulmonary hypertension, sickle cell disease, transplant rejection, and vascular injury (Szabo 2010). Clinically, the inhalation of NO gas has been approved for primary pulmonary hypertension in newborns. However, low-molecular weight NO donors rapidly scatter and are quite difficult to manipulate for targeting specific tissues. Macromolecular-derived NO release offers numerous advantages including improved storage and sustained release kinetics. Furthermore, the scaffolds may be altered with ligands for efficient targeting and/or fine-tuning of biocompatibility and clearance (Carpenter and Schoenfisch 2012). To date, several clinical trials have verified the therapeutic potential of NO donors as pharmacologically active substances (Cebova et al. 2016; Wang et al. 2002). Nevertheless, further studies designed to reveal the complex influence on vascular cell homeostasis are needed to find the key to the successful therapeutic use of NO donors (Cebova et al. 2016). As previously mentioned, nitrate tolerance constitutes a limiting factor for the clinical use of NO donors. Therefore, approaches are being pursued to develop new NO donors that possesses innate NO activity, without this limitation. Potent NOS inhibitors are also the focus of current research on treating diseases that involve high concentrations of NO; e.g., arthritis, asthma, cerebral ischemia, Parkinson's disease, neurodegenerative diseases, and seizures (Serafim et al. 2012).

## 9 Summary and Future Direction

The gaseous molecules, CO, H<sub>2</sub>S, and NO can act individually, but also can compete or synergize at the same protein target. The subtle, albeit complex, behaviors of CO, H<sub>2</sub>S, and NO are critical in a variety of fundamental homeostatic mechanisms, which become dysregulated in numerous disease processes. In this review, the cardiovascular roles of CO, H<sub>2</sub>S, and NO were briefly addressed in view of their binding either to heme-containing proteins or to non-heme iron-containing proteins, resulting in target activation or inhibition, controlling ion channel activity (Fig. 2 and Table 2), or mitochondrial respiration, either directly or indirectly (Fig. 3). Considering the promising preclinical results concerning the cytoprotective and vasodilatory effects of gasotransmitters (CO, H<sub>2</sub>S, and NO) under controlled conditions, clinical use of these agents should be considered for treating hypertension, angiogenesis, I/R injury (i.e., acute myocardial infarction and stroke), and as a supplementary therapy for the transplantation of various organs in the near future (Calvert and Lefer 2010).

There is abundant evidence that CO, H<sub>2</sub>S, and NO play critical roles in the cardiovascular system by regulating various signaling molecules involved in the sGC–PKG pathway, and influencing ion channel activities and the mitochondrial respiratory chain, although numerous unresolved questions exist. Experiments using one or two signaling agents may greatly oversimplify the situation and underestimate the overall effects and roles of each species in an integrated system, owing to possible interconnectivity among CO, H<sub>2</sub>S, and NO (Thomas et al. 2015). It remains unclear whether CO, H<sub>2</sub>S, and NO exert these actions only through the simple gas state, or whether other forms such as ionized molecules or gas molecules bound to transport proteins are involved. Unlike the gaseous form, the ionized form or the protein-bound forms do not easily penetrate the lipid bilayer, and their actions are likely confined to the site of production. Occasionally, discrepancies in pharmacodynamic effects seen with the endogenous forms compared to the exogenous forms of CO, H<sub>2</sub>S, or NO have been found when acting at the same target. For example, endogenous H<sub>2</sub>S produced enzymatically can regulate basal lipid phosphatase PTEN activity via S-sulfhydration, whereas exogenous administration of NaHS cannot (Traylor and Sharma 1992). Regarding the therapeutic effects of CO-, H<sub>2</sub>S-, and NO-releasing prodrugs in a preclinical setting (Tables 3, 4, and 5), it is not clear how the identified therapeutic actions of pro-drugs can be accurately associated in an *in vivo* system, because gaseous molecules that are released from the pro-drug will be rapidly trapped by hemoglobin or myoglobin, which is abundant in the blood. Therefore, this raises the possibility that gaseous molecules can be released from their hemoglobin- or myoglobin-bound states under unknown conditions, and to undefined degrees, which could then allow the gas molecules to exert effects on distant target sites *in vivo*. However, confirming this mechanism will require more extensive work.

Exploring the relationship and interactions among different gasotransmitters may improve clinical translation intended to maximize their beneficial actions, while

reducing adverse effects. Beyond the ion channel-modulatory effects of these gases, the mitochondrial biogenesis-stimulating effects of CO, H<sub>2</sub>S, and NO and their control of mitochondrial respiration (Fig. 3) will serve as valuable tools that provoke extensive investigations in improving mitochondrial health to treat cardiovascular complications associated with mitochondrial disorders. The CVS constitutes another important communication route in the body, and thus dysregulation of this system will cause subsequent multi-organ dysfunction. In this regard, health maintenance and/or promoting the effects of exercise may stimulate the energetic status of multiple organs (Vega et al. 2017; Kokkinos 2014). In addition, exercise is widely viewed as a low-cost and physiological booster for the human body that can suppress muscle wasting and improve the flexibility of the CVS (Leitner et al. 2017). Although only speculative, it may be meaningful to determine whether targeted applications of CO-, H<sub>2</sub>S-, and NO-donors mimic the beneficial role of exercise, or whether exercise may cause changes in CO-, H<sub>2</sub>S-, and NO-producing systems.

An understanding of the relationship between individual gas levels and their pharmacodynamic effects (e.g., cytoprotective or cytotoxic) is difficult to obtain, considering that the majority of studies that have described cytoprotective and cytotoxic roles for CO, H<sub>2</sub>S, and NO gas under pathologic conditions have often been performed using exogenous applications of each gas or through a gas-releasing donor (Tables 3, 4, and 5), or because the phenotypes were obtained by genetic manipulation (i.e., knock-out or transgenic animals) (Kajimura et al. 2010). Numerous findings from murine model systems have provided a good foundation but are lacking in terms of clinical relevance owing to the existence of endogenous differences in the anabolic and catabolic pathways of these molecules (Polhemus and Lefer 2014). The endogenous amounts of all three gasotransmitters, CO, H<sub>2</sub>S, and NO, may increase or decrease under various disease conditions. Many new technologies, that can detect the physiological levels of CO, H<sub>2</sub>S, and NO in vivo in a real-time manner, with reasonable spatial information, are required to evaluate the exact physiological and pathological roles of these gasotransmitters (Kajimura et al. 2010). In addition, a reliable serum or plasma marker for CO-, H<sub>2</sub>S- and NO-producing enzymes, such as HO-1, CBS, CSE, and NOS, will be an important and useful addition in the future, because it will help with a clinical assessment of the role of these enzymes in cardiovascular diseases (Wang and Chau 2010). With respect to the issue of gas levels, humans are not confined to a controlled diet, unlike laboratory animals. Thus, nutritional aspects should also be another concern examined in gasotransmitter studies because nutritional components containing L-arginine, L-citrulline, and nitrate for NO; L-cysteine and polysulfides (e.g., garlic) for H<sub>2</sub>S; or meats that are rich in heme proteins for CO can affect the basal production and/or regulation of those gasotransmitters (Szabo 2010). In addition, it should be remembered that significant differences may exist in the production and metabolism of CO, H<sub>2</sub>S, and NO between human and laboratory animals, as well as between individuals. In biological systems, numerous enzymes do not perform a single task, but are also involved in signal transduction regardless of enzymatic activity, as has been shown for NO-mediated GAPDH-Siah signaling (Hara and Snyder 2006). Given this, enzymes involved in the production of CO, H<sub>2</sub>S, and NO may exert different

effects through assembly with other protein complexes or movement to different cellular compartments. We speculate that both the localization and distribution of gas-synthesizing enzymes, as well as their interaction with other proteins, will be of importance, because subcellular targeting and protein interactions are strongly involved in controlling both upstream and downstream signaling initiated by specific gas molecules. Unfortunately, the elucidation of the mechanisms of gas-producing enzymes is not a simple task, although this aspect still merits investigation. Both iNOS and HO-1 are inducible enzymes that are controlled by genetic regulation. The question remains as to whether an inducible form of an H<sub>2</sub>S-synthesizing enzyme exists. If not, it is unclear why biological systems do not require it.

Taken together, the literature reveals that CO, H<sub>2</sub>S, and NO are not just simple metabolites, but rather constitute subtle endogenous regulators governing homeostasis and conferring cellular protection against pathological conditions in the CVS. Understanding the individual and concerted actions of these gaseous molecules will expand our insights into mechanism involved in the pathogenesis of cardiovascular disease. The rapid action of these gaseous molecules in biological systems is an attractive aspect in developing pro-drugs with more targeted activities and fewer adverse effects. The more we understand the biology of these gaseous molecules, the better chance we have of obtaining tools for improving human health.

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*Conflicts of Interest*

The authors declare that there are no conflicts of interest.

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