Review article

Redox regulation of gasotransmission in the vascular system: A focus on angiogenesis

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ABSTRACT

Reactive oxygen species have emerged as key participants in a broad range of physiological and pathophysiological processes, not least within the vascular system. Diverse cellular functions which have been attributed to some of these pro-oxidants within the vasculature include the regulation of blood pressure, neovascularisation and vascular inflammation. We here highlight the emerging roles of the enzymatically-generated reaction oxygen species, O2⁻ and H2O2, in the regulation of the functions of the gaseous signalling molecules: nitric oxide (NO), carbon monoxide (CO), and hydrogen sulphide (H2S). These gasotransmitters are produced on demand from distinct enzymatic sources and in recent years it has become apparent that they are capable of mediating a number of homeostatic processes within the cardiovascular system including enhanced vasodilation, angiogenesis, wound healing and improved cardiac function following myocardial infarction. In common with O2⁻ and/or H2O2 they signal by altering the functions of target proteins, either by the covalent modification of thiols or by direct binding to metal centres within metalloproteins, most notably haem proteins. The regulation of the enzymes which generate NO, CO and H2S have been shown to be influenced at both the transcriptional and post-translational levels by redox-dependent mechanisms, while the activity and bioavailability of the gasotransmitters themselves are also subject to oxidative modification. Within vascular cells, the family of nicotinamide adenine dinucleotide phosphate oxidases (NAPDH oxidases/Noxs) have emerged as functionally significant sources of regulated O2⁻ and H2O2 production and accordingly, direct associations between Nox-generated oxidants and the functions of specific gasotransmitters are beginning to be identified. This review focuses on the current knowledge of the redox-dependent mechanisms which regulate the generation and activity of these gases, with particular reference to their roles in angiogenesis.

1. Introduction

The vascular system represents a closed circuit of vessels that collectively transports oxygen and nutrients to all tissues. This delivery system has evolved multiple mechanisms that allow it to adapt to dynamic changes in tissue physiology in order to ensure that the supply of oxygen and nutrients matches the tissue demand at any given time [1]. One way in which this is achieved is through the growth of new blood vessels from the existing vasculature in a multistep, highly coordinated process known as angiogenesis. In this process, activated endothelial cells (from either arterial or venous vessels) release proteases which degrade the underlying basement membrane and subsequently migrate into the extracellular matrix. Adjacent endothelial cells in turn proliferate, differentiate and form luminal tubes which eventually fuse to the pre-established vascular network [2]. These adaptive responses are biochemically coordinated by a number of cell signalling mediators, most notably the vascular endothelial growth factor (VEGF) family of protein growth factors [3]. VEGF targets the endothelium to initiate angiogenesis and comprises multiple isoforms of which VEGF-A is a key regulator of vessel growth which has been shown to induce endothelial cell growth and survival both in vitro and in vivo [4–6]. The central role of VEGF-A in physiological angiogenesis (and vasculogenesis) has been demonstrated during embryonic and early postnatal development [6] as well as in pathological angiogenesis seen in solid tumour formation [7]. At the biochemical level, the effects of VEGF are mediated via its binding to
including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (•OH). As oxygen is reduced various reactive oxygen species are generated in the process enzymatically generated within vascular cells [13]. The precise mechanisms of the regulation of action of these short-lived mediators, which comprise nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H$_2$S) are not currently fully understood. However, there is growing evidence that their generation may be regulated in part by redox-dependent mechanisms, while their chemical nature in some cases makes them highly susceptible to oxidation. In this review we summarise the current knowledge of the biochemistry which links reactive oxygen species generation, redox signalling and the action of the gasotransmitters in angiogenesis. A more comprehensive understanding of these mechanisms would be of great potential benefit in identifying new therapeutic targets for both cancer and vascular diseases such as peripheral arterial disease (PAD) [14].

### 1.1. Reactive oxygen species and redox-signalling

Reactive oxygen species are partial reduction products of molecular oxygen (O$_2$) and include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (•OH) (Fig. 2). Historically, they have been thought of as merely potentially detrimental by-products of aerobic metabolism in the mitochondria or the result of unregulated uncoupling of various O$_2$-dependent enzymatic reactions [15]. The harmful biological effects of these oxidants are countered by the actions of enzymatic and non-enzymatic antioxidants that collectively form the cellular antioxidant system. Enzymatic antioxidants include superoxide dismutase (SOD) that removes O$_2^-$, as well as catalase, peroxiredoxin and glutathione peroxidase (GPx) that metabolise H$_2$O$_2$ [16]. Non-enzymatic antioxidants include vitamins C and E as well as the major redox buffer, glutathione (GSH). GSH is present at millimolar concentrations in the cell and scavenges both H$_2$O$_2$ and free radicals through the formation of oxidised GSH (GSSG). The “steady state” cellular redox status is maintained through the balance of constitutively expressed pro-oxidants and these antioxidant systems (reviewed in [17]).

However it is now understood that physiological, enzymatic sources of H$_2$O$_2$ and O$_2^-$ exist which generate these species in a tightly regulated and localised fashion. Moreover, the short-lived, regulated production of these pro-oxidants can mediate various homeostatic aspects of intracellular and extracellular function [18,19]. Both O$_2^-$ and H$_2$O$_2$ are capable of orchestrating a variety of cell signalling responses that culminate in a broad range of phenotypic outcomes such as altered proliferation, adhesion and invasion of endothelial cells. Thus they can act as intracellular second messengers in various signalling pathways to control cell function. Due to differences in the chemical properties of each type of oxidant species, such as half-life and lipid solubility, not all reactive oxygen species are able to function as efficient signalling mediators [20]. For example, O$_2^-$ has a short half-life of only 1 μS and is...
Fig. 3. H$_2$O$_2$-induced post-translational modifications. A schematic diagram indicating H$_2$O$_2$-induced post-translational modifications of a cysteine thiol moiety. The pKa, which is a measure of hydrogen dissociation from a given thiol, is determined by the microenvironment in which the thiol is situated, with basic surrounding amino acids tending to deprotonate the thiol more efficiently than acidic amino acids A) The progressive oxidation of a deprotonated, reactive thiol by H$_2$O$_2$ proceeding through sulphenic, sulphonic and sulphonic acid states. B) Inter and Intra-molecular disulphide bond formation. These modifications lead to functional changes within a protein.

membrane-impermeable due to its polarity. Therefore its signalling capacity is limited. By contrast, H$_2$O$_2$ is more stable and has a relatively long half-life of 10 µS. It can freely permeate biological membranes and has emerged as the major redox metabolite mediating redox signalling events [21].H$_2$O$_2$ acts to modulate numerous cellular processes via diverse mechanisms including the regulation of gene transcription, mRNA and/or protein stability, intracellular trafficking and protein activity [22]. The precise mechanisms which underlie the regulation of the generation of H$_2$O$_2$ and the specificity of its action are not fully understood. However it is clear that in complex metazoan systems, the modulation of redox-signalling events must include intracellular compartmentalisation and gradients of the oxidative signal, together with differential reactivity of the target sensors and bioavailability of buffering thiol peroxidase systems [21,22].

Physiological redox signals are transmitted though the reversible oxidative modification of target proteins to alter conformation and function.H$_2$O$_2$ can act to modify cysteine residues on redox-sensitive proteins [23] that possess reactive thiol groups which exhibit a low pKa (Fig. 3). A number of different types of H$_2$O$_2$-induced, post-translational modifications exist, including those that involve the sequential oxidation of a thiol which proceeds through sulphenic acid (SOH), sulphinic acid (SO$_2$H) and sulphonic acid (SO$_3$H) states (Fig. 3). In addition to these modifications, H$_2$O$_2$ can also induce covalent intra- and inter-disulphide bond formation to elicit a functional change (Fig. 3) [25,26]. Of these oxidation states, cysteine-sulphenic acids and disulphides are readily reversible via the cell's intrinsic glutathione and thioredoxin antioxidant systems, while cysteine-sulphonic acid derivatives, (once thought to be irreversible), can also be reduced the action of sulfiredoxins [27]. By contrast, cysteine sulphonic acid modifications are not known to be reversible. Both O$_2^-$ and H$_2$O$_2$ can also react with metal centres, often located within the active sites of target proteins. For example, H$_2$O$_2$ can react with a Mn-Fe centre in the active site of protein phosphatase-1 leading to its inhibition [28].

1.2. Functional vascular sources of reactive oxygen species: NADPH oxidases

Within vascular cells, O$_2^-$ and H$_2$O$_2$ is derived from a number of enzymatic sources including the mitochondrial electron transport system, cytochrome p450, xanthine oxidase and uncoupled nitric oxide synthase (NOS). As stated above, the O$_2^-$ derived from these sources is generally considered to be the result of mis-regulated metabolic functions, although there is some evidence for its potential functional importance in some cellular signalling pathways. By contrast, the family of flavoenzymes known as Nicotinamide Adenine Dinucleotide Phosphate Oxidases (NAPDH oxidases/Noxs) generate O$_2^-$ or, in some cases, H$_2$O$_2$ in a tightly regulated manner as their sole biological function [29]. The prototype NADPH oxidase, originally identified in phagocytes, comprises the membrane-associated catalytic gp91phox (now termed Nox2) and regulatory p22phox subunits, in addition to the cytosolic subunits; p47phox, p67phox and p40phox together with the small GTPase, Rac1. Nox homologues were subsequently identified in non-phagocytic cells, and in humans, NADPH oxidases now comprise a known family of 7 multi-subunit transmembrane proteins (each containing a distinct catalytic subunit; Nox1-5 or duox 1 and 2) which display distinct cell-type and subcellular expression patterns [29,30]. Each catalytic Nox subunit contains at least 6 transmembrane alpha helices and, with the exception of Nox5 and duox1/2, all isoforms associate with p22phox. However, different Nox isoforms additionally associate with other varied regulatory proteins which modulate their regulation and function. For example, p22phox can associate with Polymerase Delta Interacting Protein 2 [31] to activate Nox4 in smooth muscle cells (SMCs) whereas Nox1 activation is mediated by association with Nox organisier 1 (NOXO1) and Nox activator 1 (NOXA1) [32]. Mechanistically, all the Nox subunits shuttle electrons from NADPH down an electrochemical gradient across a membrane to molecular oxygen (O$_2$) which is subsequently reduced to superoxide (O$_2^-$) [33,34]. However, in the case of some Noxs (such as Nox4 and duox1/2) the O$_2$ that is produced is very rapidly converted to H$_2$O$_2$. The exact mechanism(s) by which this is achieved is still unclear although some studies suggest that Nox4 may be capable of mediating an intrinsic superoxide dismutase activity [35]. O$_2^-$ or H$_2$O$_2$ newly-generated by Noxs can be utilised by the cell in order to elicit tightly-controlled, cellular responses [29,30,36]. Moreover, the differences in structure, activity, expression, regulation and the type of oxidant species produced enable each specific Nox protein to direct its own distinct and defined functions [37–39].

Nox proteins are expressed in all of the component cell types of the vascular wall. SMCs express Nox1, Nox4 and Nox5 whereas the endothelium contains 4 Nox isoforms; Nox1, Nox2 (including the associated phagocytic subunits), Nox4 and Nox5 [40]. Their distinct physiological (and pathophysiological) roles in vascular function have, in some cases, been demonstrated in mouse models in vivo. For instance, genetic ablation of Nox1 or Nox2 was shown to elicit beneficial effects on hypertension and vascular dysfunction in certain pathophysiological settings [41–45]. By contrast, deficiency of Nox4 in mice acted to promote angiotensin II-dependent vascular dysfunction, suggesting that Nox4-generated H$_2$O$_2$ plays a protective role in vascular cells [46]. The different functions of Nox2 and Nox4 within the vasculature have been further illustrated by the contrasting phenotypes of transgenic (TG) mice which overexpress these genes specifically within endothelial cells. Thus we demonstrated that endothelial-specific overexpression of Nox2 (directed by the Tie2 promoter) resulted in increased blood pressure in mice after angiotensin II infusion, and attenuated acetylcholine- (Ach)-induced vasorelaxation in isolated aortas [47]. Meanwhile Tie2-mediated overexpression of Nox4 within endothelial cells acted to reduce basal blood pressure within TG mice, and enhanced endothelial-dependent vaso dilation ex-vivo [48]. Therefore Nox4 is increasingly considered to be a positive regulator of vascular homeostasis [48,46,49], and in this regard it may be significant that it is abundantly
expressed in the endothelium, compared to other Nox isoforms [39,50,51].

1.3. Reactive oxygen species and redox signalling in angiogenesis

There is now a large body of evidence which demonstrates that both O₂⁻ and H₂O₂ participate in redox-dependent signalling pathways which can modulate angiogenic responses both in vitro and in vivo. Early in vitro studies demonstrated that topical application of H₂O₂ to bovine thoracic aortic endothelial cells (BAEcs) enhanced proliferation, migration and tube formation compared to controls and that these effects could be ablated by the administration of catalase [52]. Perhaps significantly, it has been reported that these H₂O₂-induced angiogenic responses occur in a biphasic manner, with low concentrations of H₂O₂ enhancing angiogenic phenotypes and higher concentrations inhibiting them [53]. In addition, as might be expected from their very diverse properties, there is specificity with regard to the angiogenic effects of individual reactive oxidant species. For example, O₂⁻ and H₂O₂ were shown to be both pro- and anti-angiogenic at varying concentrations, whereas •OH was found only to exert anti-angiogenic effects, or to have no effect at all at low concentrations [53].

Many studies have now shown that some reactive oxygen species can promote the induction of VEGF expression in both endothelial and smooth muscle cells [54,55]. In addition, VEGF (and other angiogenic growth factors such as angiotropin-1) have been shown to elicit via angiogenic cellular processes are potentially varied, and there is potential function for Nox4 in the upstream activation of VEGF expression has also been demonstrated, as siRNA-mediated inhibition of Nox4 expression in an H₂O₂-dependent manner [56]. VEGF-induced endothelial cell sprouting, mediated via Transforming growth factor-β-activated-kinase 1 (TAK1), was also shown to involve the increased expression of (mitochondrial-expressed) SOD2. The functional involvement of H₂O₂ (generated by SOD2) was demonstrated as the impaired angiogenesis, observed in aortic rings in which endothelial TAK1 was ablated, could be rescued by the overexpression of SOD2 [57]. Thus reactive oxygen species are involved in the regulation of cellular angiogenic responses, both upstream and downstream of the induction of VEGF expression. In vivo, a potential role for H₂O₂ in angiogenesis was first demonstrated in extra-cellular-SOD-transgenic mice, which displayed increased H₂O₂ production and angiogenesis in a murine model of hind-limb ischemia [58]. Moreover, increased H₂O₂ VEGF production and Akt phosphorylation, associated with the promotion of physiological pulmonary angiogenesis, was also observed in exercise-trained rats [59].

1.4. Vascular NADPH oxidases and angiogenesis

The physiological sources of reactive oxygen species which regulate angiogenic cellular processes are potentially varied, and there is evidence for the involvement of O₂⁻ and H₂O₂ derived from several different sources in some in vitro settings. For instance, the stimulation of human retinal endothelial cells with high levels of glucose increased VEGF expression and cell proliferation in a manner dependent on mitochondrial O₂⁻ production from the electron transport chain (ETC) [60]. Moreover, a recent study demonstrated that treatment of human umbilical vein endothelial cells (HUVECs) with VEGF significantly elevated mitochondrial H₂O₂ production and subsequent cell migration [61]. In addition, the inhibition of endogenous xanthine oxidase was shown to reduce VEGF-stimulated Akt phosphorylation in HUVECs suggesting that xanthine oxidase may also be important for VEGF-induced endothelial cell survival [62]. However the major sources of O₂⁻ and H₂O₂ which function as signalling molecules in the angiogenic processes in vascular cells are recognised as the NADPH oxidases [56].

The (patho)physiological importance of Nox1, 2 and 4 in angiogenic processes in vivo has, in each case, been demonstrated. Global genetic ablation of Nox1 was shown to impair tumour angiogenesis in mice [63], while (again global) genetic deletion of Nox2 or Nox4 was reported to reduce blood flow recovery in ischemic mouse hindlimb models [46,64]. Nox4 expression was further shown to be required to support exercise-induced angiogenesis in mice [65]. However, in a recent study in which the consequences of the ablation of Nox1, 2 or 4 were assessed in a slow-growing mouse tumour model, only Nox4 was found to contribute positively to angiogenesis. By contrast, ablation of Nox2 had no effect, and deletion of Nox1 actually enhanced angiogenesis [66]. The significance of the apparent discrepancies in these findings is not clear, but likely reflects the different experimental models adopted. The physiological importance specifically of Nox4 in angiogenesis is further evidenced by endothelial-specific TG over-expression of Nox4 in mice which demonstrated increased angiogenesis in an ischemic hind limb model [49]. Accordingly, in vitro, Nox4 overexpression was shown to increase endothelial cell proliferation, migration and tube formation [49,67]. It is perhaps also significant that Nox4 is an inducible gene, whose transcriptional expression is activated by hypoxia [49,67]. This clearly may have relevance with regard to its potential functional role in the regulation of angiogenesis.

At the molecular level, the mechanisms which underlie the angiogenic functions of Noxs have been extensively studied in vitro. Nox-dependent upregulation of HIFα and VEGF expression via Akt and ERK1/2 activation in prostate cancer cells was demonstrated by siRNA-mediated targeting of the common p22 phox subunit [68]. In the specific case of Nox1, overexpression in NIH3T3 fibroblasts promoted VEGF and VEGFR expression in an H₂O₂-dependent manner [69]. The (mRNA and protein) expression of Nox1 was also showed to be increased by hypoxia in A549 (epithelial) cells, which in turn resulted in increased HIF expression (the transcriptional activator of VEGF), that could be blocked by catalase, or the flavoprotein inhibitor diphenylene iodonium (DPI). Both Nox1 and Nox2 are activated by the small GTPase, Rac1 [29]. AGS cells (gastric cancer cells) in which Rac1 expression had been depleted showed reduced expression of both HIF-1α and VEGF, while media conditioned by these cells inhibited cell proliferation when applied to human endothelial cells (70). A potential function for Nox4 in the upstream activation of VEGF expression has also been demonstrated, as siRNA-mediated inhibition of Nox4 expression resulted in decreased HIF-2α and VEGF promoter activity in HEK-293 fibroblasts [71]. In addition, overexpression of Nox4 in human microvascular endothelial cells (HMVECs) was sufficient to increase VEGF mRNA levels, while downregulation of Nox4 decreased the level of (HIF-1α-dependent) VEGF expression after insulin stimulation [72].

There are also many reports of the involvement of Noxs in the signalling pathways downstream of VEGF and other pro-angiogenic growth factors. VEGF and angiotropin-1 have been shown to stimulate endothelial cell migration in a Nox2-dependent manner [73,74]. In addition, Nox4 was shown to mediate VEGF-dependent angiogenic responses in human microvascular endothelial cells in vitro, by enhancing receptor tyrosine kinase phosphorylation and the activation of ERK1/2 [75]. Moreover in a separate study, VEGF stimulation resulted in a physical interaction between phosphorylated VEGFR2 and Nox4 that promoted STAT3-dependent proliferation of endothelial cells [76]. Finally, a study by Evangelista et al. demonstrated that VEGF induced endothelial migration in a Nox4- and Nox2-dependent manner and that this involved the S-glutathiolation of SERCA2b which subsequently increased Ca²⁺ influx into endothelial cells [77]. The precise protein targets of Nox-generated O₂⁻ and/or H₂O₂ which are susceptible to oxidation and act to modulate the angiogenic responses in endothelial cells are not known. However it has been demonstrated that H₂O₂ produced by ectopic expression of extracellular SOD (ecSOD) can increase VEGF-induced phosphorylation of VEGFR2 via the oxidative inactivation of protein tyrosine phosphatase 1B (PTP1B) (a negative regulator of VEGFR2 signalling) [58]. It is of note that in many separate studies PTP1B has also been shown to be a target of oxidative modification by Nox4 [78,79]. In addition to PTP1B, VEGFR2 has also been shown to have redox-sensitive cysteine residues in its kinase domain [80] and it has been suggested that H₂O₂ can induce the formation of an inhibitory intramolecular disulphide bond in VEGFR2.
[81], However the functional significance of this with respect to the cellular angiogenic responses to H₂O₂ remains to be determined. A central principle in intracellular redox-signalling mechanisms is the localization of the (freely diffusible) signalling molecules close to their targets. The enzyme complexes generating these signals (such as H₂O₂-generating Nox4), must therefore be localised to specific intracellular compartments in order to effect specific responses. The subcellular location of Nox4 has been the subject of some controversy, and has been reported, in disparate cells types, in the nucleus, endoplasmic reticulum (ER), mitochondria, focal adhesions and plasma membrane (reviewed in [82]). The underlying reasons for these discrepancies may reflect cell-type specific differences in Nox4 function, or the poor specificity of available antibodies. However, in the case of the Nox4-dependent oxidation of PTP1B, (described above), it has been shown that the co-localization of Nox4 and PTP1B within the ER of endothelial cells was a functional requirement [78].

1.5. Gasotransmitters

It is now known that in addition to the involvement of reactive oxygen species (highlighted above) another group of endogenously-produced small molecules appear to be important for vascular responses both in vitro and in vivo, including angiogenesis, cell survival and the regulation of vascular tone [83–85]. Collectively termed “gasotransmitters”, NO, CO and H₂S comprise a group of small molecule signalling agents which were previously known as toxic gases [86] but are now recognised as physiological regulators and effectors in diverse biological processes. They have become the subject of much research and are now recognised to contribute to signalling pathways in angiogenesis both upstream and downstream of VEGF (and other angiogenic growth factor) expression as described below.

The term gasotransmitters is, however, somewhat misleading as these molecules are completely soluble at physiologically-relevant concentration and pH and so perhaps should not be considered to be gases in these circumstances. None-the-less, their discovery and the characterisation of their mechanisms of action has altered the conventional paradigm of intercellular signalling. Unlike conventional signalling molecules (such as peptides) that are often stored in vesicles, gasotransmitters are generated on demand by distinct enzymes. Furthermore, peptide signals tend to mediate their function by binding to plasma membrane receptors such as receptor tyrosine kinases. By contrast, gasotransmitters can diffuse across membranes both within and between cells and subsequently interact with their protein target(s) directly to modify structure and function [84]. In this regard they share similar properties with some reactive oxygen species, most notably H₂O₂. Their biochemical targets (in common with both O₂⁻ and/or H₂O₂) are redox metal centres (most notably iron-containing haem proteins) and redox-active amino acids such as cysteine thiol groups. Chemical reactions between the gasotransmitters and some reactive oxygen species (such as the reaction between O₂⁻ and NO to form peroxynitrite (NOO⁻)), together with significant commonality in their targets, results in a significant interdependence between these groups of signalling molecules in their biological functions [87]. Thus the specific chemical properties and reactivity of the signalling oxidant molecules and the gasotransmitters have been utilised together to modulate complex signalling pathways as is beginning to become apparent.

As stated above, gasotransmitters must be synthesised, as required, close to their specific sites of action [84] and therefore both the expression and the activity of their biosynthetic enzymes must be tightly controlled. Within the vascular system, the relevant enzymes involved in the generation of these gases are endothelial nitric oxide synthase (eNOS) for NO [88], haem oxygenase-1 (HO-1) for CO [89] and cystathionine gamma-lyase (CSE) for H₂S [90] (Fig. 4). In addition to the modulation of function of the gasotransmitters by their direct reactivity (in some cases) with O₂⁻ and/or H₂O₂, there is also increasing evidence that the expression and/or function of each of these biosynthetic enzymes is regulated by redox-dependent mechanisms. Therefore the gasotransmitters should, as a group, be considered to be important effectors of redox-dependent signalling mechanisms which regulate the angiogenic cellular responses, as detailed below.

1.6. Nitric oxide (NO)

In 1980, Furchgott and Zawadzki published their seminal research on the function of the endothelium in vasomotion. Here it was demonstrated that an intact endothelium is absolutely required for the mediation of ACh-induced vasorelaxation in rabbit aortic rings [91]. These observations defined a role for the endothelium in the production of a vasoactive substance termed endothelial-derived relaxant factor (EDRF) [91] which was subsequently identified as NO [92]. Since then the study of signalling by NO has expanded exponentially and many important physiological roles within the cardiovascular system have been ascribed to this gaseous mediator [93].

NO is synthesised enzymatically by a family of enzymes collectively termed nitric oxide synthases (NOS). The NOS family encompasses three distinct isoenzymes encoded by separate genes that include neuronal NOS (nNOS, NOS-I), inducible NOS (iNOS, NOS-II) and endothelial NOS (eNOS, NOS-III). eNOS is constitutively-expressed within endothelial cells [94] and utilises the amino acid L-arginine as a substrate for the production of NO and L-citrulline under physiological conditions [95]. Numerous agonists have been shown to induce eNOS activation, including ACh, serotonin, PPARs, bradykinin, histamine, Ca²⁺ ionophores, adiponectin, VEGF, fluid shear stress and hypoxia [96,97]. NOS enzymes comprise an N-terminal oxygenase domain with binding sites for haem, L-arginine and tetrahydrobiopterin (BH₄), and a reductase domain which binds NADPH, flavin mononucleotide (FMN), FAD, and calmodulin (CaM) (reviewed in [96]). The active eNOS enzyme is a homo-dimer containing a zinc ion, tetrahedrally co-ordinated to cysteine residues at the surface of the dimer [98]. Under quiescent conditions it is anchored to caveolae through its interaction with caveolin-1, and its activation is dependent upon Ca²⁺/CaM, which binds to and displaces eNOS from caveolin, resulting in a conformational change that promotes the movement of electrons from NADPH to the haem moiety in the reductase domain [99]. Its activity can be modulated by numerous posttranslational modifications including phosphorylation at multiple serine, threonine and tyrosine sites [96], palmitylation, glutathionylation, and S-nitrosylation, and association with other proteins such as heat shock protein 90 (Hsp90) [100]. The bioavailability of its substrate, L-arginine and the BH₄ co-factor are additionally crucial determinants of eNOS function. Indeed, loss of BH₄ results in eNOS uncoupling and subsequent generation of O₂⁻[101].

1.7. The role of nitric oxide in angiogenesis

Once formed in the endothelium, NO diffuses to neighbouring SMCs where it activates soluble guanylate cyclase (sGC) by binding to its haem group. sGC converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP) which then activates Protein Kinase G (PKG). PKG has a number of downstream targets that act to cause SMC relaxation [102]. The importance of eNOS in controlling vasodilation and blood pressure is highlighted by the vessel-constrictive effects of competitive eNOS inhibitors such as NG-monomethyl-L-arginine (L-NMMA) and NG-nitro-L-arginine-methyl ester (L-NAME) on endothelium-induced vasodilation in ex vivo experiments [103], and the clinical use of these inhibitors in the diagnosis of endothelial dysfunction [104]. Direct genetic evidence for eNOS-induced control of blood pressure was confirmed in mice by disruption of the eNOS gene, since these mice displayed enhanced systemic blood pressure compared to wild-type (WT) controls [105]. In addition to its well-established role in regulating vascular tone, NO has also been shown to be a mediator of postnatal angiogenesis (Table 1). Mice in which eNOS has been genetically
ablated developed normally, but display a severe form of critical limb ischemia in mouse hindlimb models [106,107], together with impaired wound healing and defective angiogenesis [108]. Both angiogenic and arteriogenic responses to hindlimb ischemia were severely blunted in eNOS⁻/⁻ mice, an effect that could be rescued by adenosinergic-mediated delivery of a constitutively-active form of eNOS [109].

There is much evidence to support NO-mediating signalling events downstream of VEGF. Thus HUVEC form capillary-like structures when stimulated with VEGF, an effect that can be blocked by the NOS antagonist, L-NAME [110]. In vivo, mice with genetic ablation of the eNOS gene (eNOS⁻/⁻) demonstrated reduced capillary growth in implanted collagen plugs, and impaired angiogenic responses to hindlimb ischemia compared to control animals, and in both cases the phenotype could not be rescued by administration of VEGF [106,111]. In addition, VEGF-induced angiogenesis was shown to be blocked by L-NAME in a rabbit cornea model of angiogenesis [112]. Mechanistically, the activation of (human) eNOS is associated with phosphorylation at Ser⁶¹⁵, Tyr⁸¹, Ser⁶³³ and Ser¹¹⁷⁷ [113–116], and has been shown to be mediated by the actions of multiple protein kinases, including Akt/PKB, PKA, c-Src and AMPK [117,118]. Activation of VEGFR2 in endothelial cells (by VEGF binding) results in the Akt-dependent phosphorylation of eNOS at Ser⁶¹⁷, while VEGF treatment of BAECs resulted in the phosphorylation of eNOS at Ser⁶¹⁷ and Ser⁶³⁵ (equivalent to human Ser⁶¹⁵ and Ser⁶³³) [116]. The kinase responsible for phosphorylation of Ser⁶¹⁷ in this study was suggested also to be Akt, while phosphorylation at Ser⁶³⁵ (of human eNOS) has recently been shown to be mediated, in response to VEGF signalling by the serine/threonine-protein kinase, Pim1 [120]. In addition, VEGF-signalling induces the c-Src-dependent phosphorylation and association of Hsp90 with eNOS [121], eNOS is additionally activated by other stimuli, including shear stress [122], which has been shown to be dependent on both Akt- and PDK-dependent mechanisms. NO is believed to promote angiogenesis by a sGC-cGMP-dependent pathway(s), although the precise mechanisms remain to be fully elucidated [123]. There is also evidence that NO can act upstream of VEGF signalling to promote angiogenesis. Thus increased NO, either administered by exogenous NO donors, or due to induction or overexpression of NOS, acted in vascular smooth muscle cells (VSMCs) in vitro to increase VEGF expression [124,125]. In addition, inhibition of NOS in vivo eliminated the increased expression of VEGF (and VEGFR-2) in electrically stimulated capillary growth in rat muscle for up to 4 days [126]. Mechanistically, NO has been shown to activate the VEGF promoter via the cis-regulatory hypoxia response element (HRE) which mediates the transactivation of VEGF transcription by HIF-1 [127]. It should be noted, however, that in an ex vivo model of balloon angioplasty in rat thoracic aortae, increased VEGF expression was inhibited by NO donors [128] and the reasons for the discrepancies in these observations is currently not clear.

In recent years it has become apparent that asymmetric dimethyla...
larginine (ADMA) and its associated metabolic enzymes dimethylargi
nine dimethylaminohydrolase I and II (DDAH I and DDAH II) can
regulate angiogenesis through their effects on NOS substrate bioavail
ability [129,130]. DDAH is an endogenous methylated form of L-
larginine that acts as a competitive inhibitor of all NOS enzymes, and is
formed from the hydrolysis of proteins that have been methylated on
arginine residues. DDAH and II are enzymes that metabolise ADMA
and therefore negate its inhibitory effects on NOS, elevating NO levels
[131]. Both in vitro and in vivo studies have demonstrated that DDAH
enzymes are involved in angiogenesis. Aortic rings isolated from DDAH
heterozygous mice (DDAH+/−) showed reduced sprouting in Matrigel.
This effect could be mimicked through the addition of DDAH to ring
explants from WT mice, suggesting that endogenous removal of ADMA
by DDAH is required for vessel growth [132]. Moreover, endothelial-
specific ablation of DDAH blunted angiogenesis both in vitro and in vivo
[133], while gene-targeted over-expression of DDAH enhanced NO
production and angiogenesis in a murine model of hindlimb ischemia
that was associated with reduced ADMA levels [134].

1.8. Regulation of nitric oxide-dependent signalling by redox mechanisms

The relationship between reactive oxygen species and the signalling
functions of NO is complex, and species-type dependent. NO bioavail
ability is considered to be a fundamental requirement for vascular
health [135]. It has long been known that O2− readily reacts with NO to
form NOO−, and so acts to reduce NO bioavailability, thereby contribut
ing to vascular disease aetiology [136]. Indeed, Nox1- and Nox2-
derived O2− is thought to inactivate NO and enhance hypertension
[42–45] and vascular dysfunction in angiotensin II-induced stress
[137]. Nox2 activity has also been shown to directly reduce NO
bioavailability leading to enhanced plaque formation in a model of
atherosclerosis [138], while detrimental effects of O2− on angiogenesis
have also been reported [139]. A more oxidising basal cellular redox
state can also act to modulate NOS enzymatic activity by direct
oxidation of the enzyme itself, via glutathionylation [140,141] or by
oxidation of its co-factor, BH4 [142]. In both cases this converts the
enzyme from a NOS to a superoxide-generating oxidase (or uncoupled
NOS), and the NADPH oxidase, Nox2 has been demonstrated to be a
potential oxidant source [143]. The responsiveness of the major
target of NO, sGC, can also be affected by the cellular redox state. Thus
its ferrous haem can become oxidised to an NO-resistant ferric haem
which is susceptible to ubiquitylation and degradation [144].

By contrast to O2−, both exogenous and endogenously-generated
H2O2 have been shown in endothelial cells in vitro to promote both NO
production and NO-dependent signalling [145] and to stimulate
angiogenic responses (proliferation and migration) which are blocked
by inhibiting eNOS [146]. Moreover, in vivo, the transgenic over-
expression of catalase in mice demonstrated that endogenous, endothe-
lial-expressed H2O2 mediates the upregulation of eNOS after exercise
[147]. In BAECs H2O2 (but not O2−) was shown to increase both the rate
of transcription and the stability of eNOS mRNA in a dose- and time-
dependent manner [148]. These increases were shown to be prevented
by both the antioxidant N-acetylcysteine (NAC), and the H2O2 scavenger,
catalase, although the precise mechanisms of transcriptional
regulation remain unknown. H2O2 was also shown to be able to
upregulate eNOS activity through the modulation of signalling path-
ways that contribute to its activation via VEGF-dependent signalling.
Thus Thomas et al. demonstrated that H2O2 could induce the phos
phorylation of eNOS at Ser1177, which is known to be necessary and
sufficient for VEGF-mediated endothelial cell migration, and this was
shown to occur via the PI3-K/Akt pathway, shown to be activated by
VEGF signalling (see above) [149]. Consistent with this, administration
of H2O2 to BAECs was sufficient to promote NO production via Akt and
Erk1/2 [150]. Independent of VEGF, H2O2 has also been shown to be a
critical intermediate mediating phosphorylation of eNOS via activation
of the G-protein-coupled receptor for ADP, P2Y1 [151]. H2O2 admin:
istration has also been shown to stimulate phosphorylation of bovine
eNOS at Tyr43 (equivalent to Tyr81 in human eNOS) in BAECs via Src
kinase, although it is unclear whether this is VEGF-dependent or
independent [117].

As stated previously, H2O2 is produced endogenously within
vascular cells from a number of enzymatic sources including, (perhaps
most notably in endothelial cells) Nox4. Indeed, Nox4 overexpression
has been shown to increase eNOS protein expression and activity in
cultured endothelial cells and to be sufficient to promote proliferation,
migration and tube formation [49]. Moreover, in vivo, endothelial-
specific Nox4-overexpressing mice demonstrated accelerated blood-
flow recovery in an ischemic hindlimb model as well as enhanced
aortic capillary sprouting. Significantly, these Nox4-induced effects
were ablated by genetic deletion of eNOS [49]. Perhaps consistent with
these observations, global genetic ablation of Nox4 in mice resulted in
an approximately 50% decrease in eNOS expression in the carotid
artery, and significantly-reduced NO production which potentially
contributed to the attenuated angiogenesis in these mice after femoral
artery ligation [46]. Taken together, these studies strongly suggest that
Nox4 is a physiological source of enzymatic H2O2 which acts to
promote post-natal angiogenic responses, at least in part, via modula
tion of the expression of eNOS and/or modulation of VEGF-mediated
activation of eNOS activity (Fig. 5). In this regard, the potential Nox4-
generated H2O2-dependent oxidative inactivation of protein tyrosine
kinases, including PTP1B and SHP2 (negative regulators of VEGFR2
signalling) may be of functional significance [78,79,152].

As mentioned above, the bioavailability of NO is also controlled by
DDAH enzymes. A recent study has suggested a role for the redox-
sensitive transcription factor, Nuclear factor erythroid 2-related factor
(Nrf2) in the coordinated regulation of both DDAH and eNOS in renal
glomerular endothelial cells [153]. Nrf2 is a basic leucine-zipper
transcription factor that is regulated by the cytoplasmic protein
Kelch-like ECH-associated protein 1 (KEAP1). In normal physiological

Fig. 5. Reactive oxygen species-dependent mechanisms regulating NO bioavail
ability. A) H2O2 (potentially generated by Nox4) can increase eNOS expression via an
unknown mechanism leading to increased NO production. B) O2− Oxidant-dependent
activation of Nrf2 leads to increased transcription of eNOS (and therefore NO production)
and DDAH. Increased DDAH expression in turn acts to reduce ADMA (a competitive
inhibitor of NOS) levels resulting in increased NO production. C) H2O2 (potentially
generated by Nox4) promotes the activation of the PI3K/Akt signalling pathway which
results in phosphorylation and activation of eNOS and increased NO production. D) O2− is
capable of reacting with NO to form peroxynitrite which reduces the bioavailability of
NO. E) Glutathionylation of eNOS under oxidative conditions and G) Oxidation of the
eNOS co-factor, BH4, leads to NO uncoupling, reduced NO production and O2−
production. The likely oxidant is O2− generated by Nox2. GSH: glutathione, GS:
glutathionylation, PC: phosphorylation, BH4: tetrahydrobiopterin, DDAH: dimethylargi
nine dimethylaminohydrolase, U.O.: unidentified oxidant.
conditions, KEAP1 sequesters Nrf2 in the cytoplasm where it is targeted for ubiquitin-mediated degradation. However, upon activation by pro-oxidants (among other stimuli), critical cysteine residues in KEAP1 are oxidised resulting in the liberation of Nrf2 and its subsequent translocation to the nucleus where it drives the expression of a battery of antioxidant and detoxifying genes [154]. The activation of Nrf2 using tert-butylhydroquinone was demonstrated to enhance Nrf2 translocation to the nucleus and subsequent DDAH and II expression [131]. This effect was lost upon Nrf2 ablation. In addition, concomitant with the decrease in ADMA levels observed upon Nrf2 activation, both eNOS expression and NO production increased (Fig. 5). It was concluded that Nrf2 upregulates eNOS as well as DDAH enzymes to potentiate NO production and improve endothelial cell function [153]. The physiologically relevant endogenous source(s) of the oxidant(s) which might activate Nrf2 in this process is not known. However, we have shown that increased Nox4 expression can act to promote the expression of several Nrf2-target genes in other in vivo settings (see below) [155,156], and therefore the increase of Nox4 expression seen in response to hypoxia [49] may be of relevance here.

From these studies it is clear that both O₂⁻ and H₂O₂ play diverse roles in the regulation of NO-dependent signalling, by regulating the expression and activity of eNOS, together with the bioavailability and functional capability of NO itself, and that these regulatory mechanisms have functional consequences in angiogenic phenotypes (Fig. 5).

1.9. Carbon monoxide

CO, first denoted as a ‘silent killer’, has come to be recognised as an important physiological mediator in a broad range of biological processes and systems, perhaps most notably in the neurological and cardiovascular fields [157]. CO has high affinity for haemoglobin, and therefore its subsequent ability to reduce O₂ transport in the blood led to its initial characterisation as a toxic pollutant. However, it later became apparent that CO can be generated physiologically by a family of enzymes known as haem oxygenases (HOs) [158]. This family comprises 3 isoenzymes, HO-1 [159], HO-2 [160] and HO-3 [161], of which only HO-1 and ~2 are thought to be catalytically-active proteins. HO-1 is an inducible isofrom which has been most closely associated with CO-dependent signalling. Its expression and corresponding activity is both positively and negatively regulated by a number of stimuli including hypoxia [162,163], heat shock [164] and oxidative stress [165]. It is noteworthy that these stimuli have species-specific effects, with hypoxia upregulating HO-1 in rat tissue [163] but decreasing it in human cells [162]. HO-1 has been shown to be localised to caveolae and cytosolic compartments of endothelial cells [166] as well as the nucleus under conditions of “oxidative stress” [167]. By contrast, HO-2 is constitutively expressed and is responsible for basal CO production primarily in the brain and cardiovascular system [168]. Enzymatically, HO catalyses the rate-limiting step in the breakdown of haem into biliverdin, iron and CO in a reaction that requires oxygen and NADPH [169] Once formed, CO can subsequently induce a number of targeted effects as discussed below.

1.10. The role of carbon monoxide in angiogenesis

A number of vascular roles have been ascribed to CO including reduced platelet activation [170] and monocye-induced inflammation [157]. In addition, CO has been implicated as a vasoactive mediator that can induce vasorelaxation, an effect which has been characterised in a number of vessels including rat aorta [157] as well as mesenteric, renal and pulmonary arteries [171] and has been demonstrated using both CO donors and HO inhibitors [157]. The precise mechanisms by which CO-dependent signalling occurs are not fully understood. However, in common with NO, CO is a non-polar molecule which can freely diffuse across biological membranes and can bind sGC to modulate cGMP production [87]. In addition, some groups have suggested that CO-dependent signalling in the vasculature occurs in an endothelium-dependent manner [172]. Therefore there is likely to be a great deal of interdependence between the vascular functions of CO and NO.

In addition to vascular tone, HO/CO-signalling has also been implicated in angiogenic control (Table 1). HO-1 expression is rapidly induced in bovine aortic endothelial cells in response to hypoxia [173], and early evidence for its functional significance came from studies in which HO-1 overexpression enhanced endothelial cell proliferation [174]. By contrast, targeted gene silencing of HO-1 in endothelial cells reduced capillary formation and cell proliferation. Re-introduction of CO in the form of the CO donors, tricarbonyl-dichlororuthenium (II) dimer (CORM-1) and tricarbonylciblor(glucinato)ruthenium (II) (CORM-3), was sufficient to recover the angiogenic properties of HO-1 deficient endothelial cells [89]. CO-dependent signalling has been reported to act both upstream and downstream of VEGF induction in the angiogenic response, although it should be noted that there remain inconsistencies within the literature with regard to its pro- or anti-angiogenic effects [175–177]. This may reflect the differences in cell types and/or differences in levels of CO applied to the cells [178].

Upstream of VEGF-signalling, HO-1 overexpression or activation has been shown to increase VEGF production in VSMCs, macrophages and endothelial cells [177,179] and accordingly treatment of endothelial cells with CORM was shown to mimic this effect [177]. Consistent with this, inhibition of HO activity by tin protoporphyrin IX (SnPPIX) abrogated the increase in VEGF expression in VSMCs, observed upon stimulation by hypoxia, IL-1β or hemin [180], while ablation of HO-1 was shown to inhibit VEGF expression and the angiogenic responses of endothelial cells [89]. By contrast, in a separate study, the hypoxic induction of VEGF in rat aortic smooth muscle cells was shown to be suppressed by both CO and NO [181] in a sGC- and cGMP-mediated pathway that resulted in decreased HIF-1 binding to the VEGF enhancer. Downstream of VEGF signalling, CO has also been reported to promote both pro- and anti-angiogenic effects. Thus VEGF was shown to induce sustained HO-1 expression and activity in cultured endothelial cells, and the angiogenic responses induced by VEGF were perturbed when HO-1 was inhibited [182]. However, in a more recent study using HUVEC, exposure to CO was found to inhibit VEGF-induced angiogenic responses [175]. Mechanistically, CO administration acted to suppress VEGF-stimulated VEGFR-2 phosphorylation and the downstream Akt phosphorylation. The discrepancies in these observations are likely to be due to differences in experimental design and remain to be reconciled. HO-1 has also been shown to mediate the pro-angiogenic effects of SDF-1, through a protein kinase C-ζ-dependent, VEGF-independent mechanism. Thus SDF-1 was shown to induce HO-1 expression in endothelial cells, and ablation of HO-1 in mice, in vivo, prevented aortic rings forming capillary sprouts in response to SDF-1 [183]. Mechanistically, SDF-1 was shown to promote the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) via HO-1 activation to promote angiogenesis. In addition to modulating the expression and associated signalling pathways of pro-angiogenic factors, HO-1 has also been shown to act to deplete the levels of the anti-angiogenic factors, soluble fms-like tyrosine kinase I and soluble Endoglin, thereby potentially further enhancing angiogenesis [184].

In vivo, a potential pro-angiogenic role of HO/CO-dependent signalling has also been demonstrated. Thus the pan HO inhibitor, zinc protoporphyrin (ZnPP) was found to block angiogenesis in solid tumours suggesting a physiological role for HO in neovascularisation [185]. In addition, the induction of HO-1 activity, or the administration of a CO-donor both acted to promote formation of new coronary arteries after myocardial infarction induced by coronary artery ligation in rats [186]. In addition HO-1 was shown to be necessary for efficient post-injury reparative neovascularization after both hind-limb ischemia [187] and cutaneous wounding in mice [188]. A critical role for HO-1 placental vascular formation during embryonic development is also recognised. Thus HO-1 expression and endogenous CO production is highly elevated in the placenta during pregnancy [189] and a partial
deficiency of maternal HO-1 results (in mice) in the restriction of the growth of both the placenta and the foetus that is due, at least in part, to impaired angiogenesis [190].

The manipulation of the HO/CO axis using pharmacological and genetic means has therefore implicated this pathway in the regulation of angiogenesis. However little is known about the exact mechanism(s) through which CO acts. As stated above, CO is believed to induce changes in protein activity primarily through its reaction with haem containing proteins [191], and both iNOS and NOS have been implicated as potential targets, again suggesting cross-talk between gasotransmitters [192].

1.11. Regulation of carbon monoxide-dependent signalling by redox mechanisms

It is well-established that pro-oxidant-generating stimuli such as UVA radiation and cadmium chloride, in addition to H2O2 per se, are capable of inducing HO gene expression [193]. Indeed, HO-1 expression has been used as a model system for studying redox-regulated gene expression in mammalian cells [194]. Administration of H2O2 has been demonstrated to enhance both HO-1 and VEGF expression in a variety of cell types, including vascular cells, while chemical inhibition of HO-1 activity in part abrogated the increase in VEGF expression, underlying the significance of this redox-regulation to angiogenesis [195]. From several studies it has become apparent that many redox-sensitive cell signalling pathways and transcription factors are involved in regulating HO-1 expression including Mitogen Activated Protein Kinases (MAPKs) such as p38 [196] and JNK [197] as well as the transcription factor Hypoxia Inducible Factor 1α (HIF1α) [163] and, perhaps most notably, Nrf2 [156]. Cigarette smoke contains a number of pro-oxidants and application of aqueous extracts to NIH3T3 cells induced Nrf2 activation leading to HO-1 induction, an effect that was lost when Nrf2 was silenced [198]. Nrf2 has been shown to induce HO-1 transcription through two distal cis-acting enhancer elements in the HO-1 promoter that both contain Nrf2-binding consensus antioxidant response elements (AREs) [198]. The regulation of HO-1 in response to oxidents is further complicated by the involvement of a hypoxia-induced repressor of HO-1 transcription termed BTB Domain and CNC Homolog 1 (Bach1) [199]. By contrast to Nrf2, the activity of Bach1 is inactivated by oxidents and it was shown in human keratinocytes that arsenite-mediated HO-1 induction involves both the removal of Bach1 from the HO-1 promoter and the subsequent Nrf2-directed up-regulation in its expression [200]. Indeed, hypoxia-dependent suppression of HO-1 in human endothelial cells appears to be regulated by a complex interplay between Bach1 and Nrf2 [162], which is in turn potentially mediated by redox-dependent mechanisms.

Although it is well accepted that the Nrf2/HO-1 response is (in part) oxidant driven, the physiological endogenous source(s) of these oxidents remains unknown. However we have shown that Nox4-generated H2O2 can activate Nrf2 and induce HO-1 expression in cardiac-specific Nox4-overexpressing hearts [155]. Moreover, in vitro simulation of hemodynamic stress, using phenylephrine-stimulated neonatal rat ventricular myocytes, resulted in an increase in both Nox4 and Nrf2 protein expression, and a subsequent up-regulation in HO-1 mRNA expression. This effect was ablated when either Nox4 or Nrf2 were silenced [156] (Fig. 6). Consistent with these observations, global Nox4-null mouse display reduced HO-1 expression in isolated lung endothelial cells, an effect that resulted in enhanced apoptosis. This has been attributed to perturbed CO production as re-introduction of CO using CORMs enhanced lung endothelial cell survival [46].

Intriguingly, there is also potential for the converse cross talk between CO- and Nox-dependent signalling mechanisms. Thus it has been demonstrated that CO administration can act to inhibit the activity of Nox2 within human airway smooth muscle cells and neutrophils [201] due to binding to the haem moiety. It will be of interest to determine whether this is a common regulatory mechanism, potentially modulating the activity of other NAPDH oxidases.

1.12. Hydrogen sulphide

H2S was considered a toxic environmental pollutant until Abe and Kimura first proposed it to be an endogenous cellular signalling mediator [202]. Subsequent extensive research efforts have established H2S as a third member of the gasotransmitter family of signalling molecules and have ascribed to it a number of diverse functions within different physiological systems including the cardiovascular, immunological and nervous systems [203]. H2S is synthesised endogenously through the action of 3 pyridoxal 5’ phosphate- (vitamin B6)-dependent enzymes, cystathionine β-synthase (CBS), CSE and the combined action of 3-mercaptopyruvate sulphur transferase (3MST) and cysteine aminotransferase (CAT) [204]. Each of these enzyme systems displays promiscuity with respect to substrate specificity and function, and can additionally catalyse other reactions in the sulphur metabolic network [205]. These enzymes also display tissue-specific patterns of expression. Thus CBS plays a major role in H2S generation in the nervous system [206], while 3MST is widely distributed in multiple cell types including neurons, hepatocytes, cardiac cells and endothelial cells [207,208]. However, within the vascular system, the major enzymatic source of H2S is CSE, which has been shown to be expressed and be active in a number of vascular cell types including SMCs [209], perivascular adipose cells [210] and endothelial cells [211–213]. CSE is a tetrameric protein and it generates H2S from a number of substrates including cysteine, cystine and homocysteine [205,214]. Subcellularly CSE and CBS have been found in the cytoplasm [215], but have also been suggested to be in the nucleus [216] and under hypoxic conditions in the mitochondria [217,218]. CAT and 3MST are generally considered to be localised to the mitochondria (see Fig. 4) [219,220].

The chemical properties of H2S are such that under physiological conditions in aqueous solutions at pH 7.4, H2S exists in a dissociated state with approximately two thirds of total H2S existing as hydro-sulphide anions (HS-) and protons (H+) with the remaining third being in the form of H2S undissociated, (dissolved) gas. HS- can subsequently form sulphide ions (S2-) through further dissociation but only at high, non-physiological pH. {S2-} is therefore considered negligible in physiological systems [221]. Undissociated H2S is a non-polar, lipophilic molecule which can diffuse through membranes in a similar manner to NO and CO. However, due to its dissociation at physiological pH, H2S should be considered to be less lipid-permeable than the other
gas transmitter molecules [222], and this may impact on its signalling functions. The term H2S is typically used collectively to denote both H2S and HS− anions as it is currently unknown which of these is the most physiologically relevant species. Once generated, H2S is believed to function as a secondary messenger by directly modulating the function of downstream targets. In common with both NO and CO, H2S can form coordination complexes with metal centres (such as haem groups) within proteins [233]. In addition, it acts to covalently modify cysteine residues on target proteins in a process known as S-sulfhydration [224]. Here, sulphur derived from H2S becomes added to the thiol group of a target cysteine residue to render the formation of a hydrosulphide moiety (SSH). In one study it was shown that as much as 10–25% of some abundant proteins in mouse liver including actin, tubulin and glyceraldehyde-3-phosphate (GAPDH) exist in the S-sulfhydrylation state under physiological conditions [224]. Further, this was shown to be mediated by H2S generated enzymatically from L-cysteine by CSE. Thus the limited number of specific H2S target proteins currently identified [224] seems likely to grow.

1.13. The role of hydrogen sulphide in angiogenesis

One of the first biological activities attributed to H2S was in the regulation of vascular tone [225]. Subsequent research efforts using a combination of H2S donors [210,226–229] and direct genetic manipulation of the CSE gene have confirmed the physiological relevance of the CSE/H2S pathway in vasorelaxation and blood pressure regulation [230]. At the cellular level, studies using patch clamp techniques have demonstrated that both endogenous and exogenous H2S can directly hyperpolarize resistance artery VSMCs via modulation of ATP-gated K+ (KATP) channel activity, and that these effects are independent of cGMP-mediated phosphorylation [231]. More recently, H2S has been demonstrated to be an endothelial-derived hyperpolarizing factor (EDHF) which exerts its vasodilatory actions on VSMCs by causing the opening of KATP channels, at least in part, through S-Sulfhydration of the Kir 6.1 subunit at cysteine-43 [212]. In addition to its actions as an EDHF acting on adjacent smooth muscle, H2S signalling is also thought to be important within endothelial cells themselves. Endothelial CSE can be activated by Ca2+/calmodulin [230] in response to cholinerigic stimulation or by the calcium ionophore, A23187 leading to enhanced H2S production [230]. Moreover, stimulation of isolated endothelial cells with ACh resulted in hyperpolarisation in cells isolated from WT but not CSE−/− mice. This effect is mediated not by KATP channels but by small and intermediate calcium-gated K+ channels present within the endothelium, as the hyperpolarisation could be blocked by apamin and charybdotoxin [212]. Whether specific cysteine residues within these calcium-gated K+ channels are targets of S-Sulfhydration remains to be demonstrated.

H2S has also been shown to be a key regulator of angiogenesis (Table 1). Thus topical application of H2S to endothelial cells in vitro increased HUVEC proliferation as well as migration and capillary morphogenesis on Matrigel, which are considered essential initiating steps in the angiogenic response [90,232]. In vivo, administration of NaHS (an H2S donor) increased neovascularisation and haemoglobin steps in the angiogenic response [90,232]. Here, sulphur derived from H2S becomes added to the thiol group of a target cysteine residue to render the formation of a hydrosulphide moiety (SSH). In one study it was shown that as much as 10–25% of some abundant proteins in mouse liver including actin, tubulin and glyceraldehyde-3-phosphate (GAPDH) exist in the S-sulfhydrylation state under physiological conditions [224]. Further, this was shown to be mediated by H2S generated enzymatically from L-cysteine by CSE. Thus the limited number of specific H2S target proteins currently identified [224] seems likely to grow.

1.14. Regulation of hydrogen sulphide-signalling by redox-dependent mechanisms

As is the case for NO and CO, the signalling functions of H2S that involve binding to metal centres can be mediated by the redox state of the coordinated metal ion, which in turn may be modulated by both the "steady state" redox potential of the cellular environment and/or the localised production of reactive oxygen species such as O2•− or H2O2. In addition, the signalling mechanisms of H2S which involve thiol modification may also be subject to redox-dependent regulation. The precise mechanism through which H2S induces its modifications remains contentious. It was thought initially that H2S attacks previously oxidised cysteine residues such as those in the sulphenic acid (SOH) or...
disulphide bonded (S-S) state in a reduction reaction [224]. However recent studies have suggested that in order to mediate its function, H2S acts as an oxidant molecule through an initial reaction with H2O2 to form polysulphides (H2Sn). These polysulphides can then oxidise cysteine residues on target proteins yielding the persulphide moiety [226,235]. Such a mechanism of H2S-mediated oxidative activation, ultimately resulting in the formation of an intermolecular disulphide bond, has been demonstrated in the case of PKG [226]. Moreover, the H2O2-dependent conversion of H2S to polysulphides results in the loss of polarity, and therefore greater lipid solubility of the signalling moiety. This therefore potentially increases the ability of H2S to signal to adjacent cells, and may be an important contributory factor in regulating the signalling functions of H2S.

Another potential level at which reactive oxygen species may act to mediate H2S-dependent signalling is by regulation of CSE-dependent H2S production. At the level of gene expression, Wang et al. showed that exogenous H2O2 increased the activity of the CSE promoter [243] while in another study CSE transcription was upregulated in rat mesangial cells by treatment with platelet-derived growth factor, and this upregulation was abolished by antioxidants including NAC and the Nox inhibitor, DPI [244]. However in the interpretation of these results it should be noted that DPI is an inhibitor of all flavoproteins, including NOS [245]. A potential role for Nox4 in the transcriptional regulation of CSE is also suggested by studies conducted in our group. Thus we have demonstrated that in endothelial cells in vitro, Nox4-generated H2O2 acts as a positive regulator of CSE transcription, via activation of a haem-regulated inhibitor kinase/sel2a/activating transcription factor 4 (ATF4) signalling module and CSE was found to be a direct transcriptional target of ATF4 [246]. In vivo, endothelial-specific Nox4 transgenic mice exhibited lowered blood pressure [48] and a hypo-contractile phenotype in response to phenylephrine that was abolished when vessels were incubated with the CSE inhibitor, proparglyglycine [246]. We therefore conclude that Nox4 may be a physiologically relevant source of H2O2 that positively regulates CSE expression and H2S production [246] in endothelial cells (Fig. 7).

In addition to playing a role in the transcriptional regulation of CSE, H2O2-dependent signalling may also modulate CSE activity. Thus in HUVECs it was shown, using a cell-trappable fluorescent H2S probe, that VEGF application acted to increase CSE-derived H2S production in a H2O2-dependent manner. The relevant source of H2O2 in these experiments was again suggested to be a Nox, as the increase in H2S production could be inhibited by DPI (although, as stated above, DPI is not a specific Nox inhibitor). Clearly, however, crosstalk between H2S and H2O2 in endothelial cells after VEGF-activation, may be important in the regulation of angiogenic cellular responses [247].

1.15. Interactions between gasotransmitters

The sequential discovery and characterisation of each of the gasotransmitters had initially led them to be considered as biologically distinct entities that mediate their functions independently of each other. While this is no doubt the case in certain settings, it has become apparent that considerable synergistic and antagonistic interplay occurs between this family of gases at multiple levels creating a multifaceted signalling platform though which key cellular and physiological processes, such as angiogenesis, are orchestrated [87]. Coletta et al. studied the synergistic effects of H2S and NO in the angiogenic response. Here, the pro-angiogenic effects of H2S were ablated in endothelial cells isolated from eNOS gene-deficient mice. Conversely the silencing of CSE and concomitant reduction in H2S production prevented NO-stimulated angiogenesis. Mechanistically, the mutual dependency between H2S and NO in the endothelium occurs on multiple levels and appears to be centred on the production of cGMP. NO is known to bind to and activate sGC leading to elevated cGMP production. By contrast H2S inhibits phosphodiesterase-5 (PDE5) preventing cGMP degradation. Furthermore H2S was also shown to enhance NO production in endothelial cells. Thus these gasotransmitters act in synergy to potentiate the effect of cGMP leading to protein kinase G (PKG) activation and subsequent modulation of key aspects of angiogenesis [123]. Interestingly, the haem group of sGC can be bound and activated by CO and NO but with varying affinities. CO, for example, binds at lower affinity than NO, an observation that may relate to compensatory actions of the gases [248]. By contrast H2S can reduce the haem iron of sGC from its ferric form to its ferrous form, an effect that promotes the binding of NO [238]. This ability for a single protein to become the target of multiple gasotransmitters has been shown to extend to a number of other proteins such as GAPDH which can be S-sulphhydrated by H2S and nitrated by NO [224].

The interrelationship between NO and CO extends further as it has been shown that NO can stimulate HO in the endothelium [249] but inhibit it in the brain [250]. To add a further layer of complexity to the interplay between the gases, it is becoming apparent that they may be able to react together to form novel signalling intermediates. Indeed the NO donor sodium nitroprusside (SNP) and H2S can react to form nitroxy (HNO) [251] which has been shown to elicit anti-angiogenic effects in tumours [252]. Taken together it is clear that the effects of these gases cannot be viewed simply in terms of their individual functions and must be considered as part of a more complex system, in which many aspects of their biology are crucial, including, for example, their subcellular location. It is conceivable that compartmentalisation of the gasotransmitter producing enzymes into the same subcellular location may facilitate the interaction of their respective gases thereby helping to fine-tune the varied signalling responses associated with them. Perhaps consistent with this notion, O2 and H2O2 (enzymatically produced in a highly-regulated, compartmentalised manner), can potentially act in a similar way through varied and complex interactions with the gasotransmitters to yield diverse signalling molecules such as polysulphide [226] and peroxynitrite [253]. A greater understanding of the chemistries and interrelated actions of these gases is now very much needed and will no doubt lead to a fuller understanding of their roles in many biological processes, including angiogenesis.

2. Conclusion and future perspectives

In the last decade the conventional paradigm of reactive oxygen species as deleterious by-products of aerobic metabolism has shifted, and now it is largely accepted that they represent a group of highly regulated and coordinated secondary messengers capable of mediating a number of physiological processes [254] including angiogenesis. In
particular, H₂O₂ has been shown to induce endothelial cell phenotypes such as increased proliferation, tube formation and migration that are all fundamental to the angiogenic response [52]. Nox enzyme complexes have emerged as the significant endogenous sources of O₂⁻ and H₂O₂ within the vasculature and Nox4, in particular, seems a likely generator of the H₂O₂ shown to be important in the angiogenic cellular response. Thus Nox4 is highly expressed in vascular cells compared to other Nox isoforms, and its expression increases in response to tissue hypoxia [67], a condition in which many pro-angiogenic factors such as VEGF are released. Secondly, by contrast to Nox1 and Nox2 which generate O₂⁻ Nox4 is considered to be a generator of H₂O₂, the oxidant species shown to be most pro-angiogenic [53]. Lastly, genetic gain- and loss-of-function models, both in vitro and in vivo, have consistently highlighted a positive role for Nox4 in ischemia-induced angiogenesis [46,49].

The roles of reactive oxygen species, notably Nox-generated O₂⁻ and H₂O₂, are increasingly complex and diverse. Moreover it is clear that their functions are intimately connected to those of the gasotransmitters, NO, CO and H₂S. The production of all these reactive signalling molecules occurs in a highly coordinated manner, close to their biological sites of action. They can also act independently, synergistically or antagonistically to elicit their downstream effects often culminating in a highly fine-tuned response. The transient chemical nature of the gasotransmitters, as well as their inherent need to be produced at their sites of action, and their toxicity in non-physiological concentrations, preclude their direct use in modulating angiogenesis therapeutically. However, the potential use of (sometimes slow-releasing) donor compounds of NO, CO and H₂S as clinical angiogenesis therapeutically. However, the potential use of (sometimes slow-releasing) donor compounds of NO, CO and H₂S as clinical therapeutics is being vigourously explored [255]. In this regard a hybrid molecule which has been shown to exhibit greater proangiogenic activity under hypoxic conditions [67], a condition in which many pro-angiogenic factors such as VEGF are released. Secondly, by contrast to Nox1 and Nox2 which generate O₂⁻ Nox4 is considered to be a generator of H₂O₂, the oxidant species shown to be most pro-angiogenic [53]. Lastly, genetic gain- and loss-of-function models, both in vitro and in vivo, have consistently highlighted a positive role for Nox4 in ischemia-induced angiogenesis [46,49].

Conflict of interest statement

None.

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