Novel Nox homologues in the vasculature: focusing on Nox4 and Nox5

Augusto C. MONTEZANO*, Dylan BURGER*, Graziela S. CERAVOLO*†, Hiba YUSUF*, Maria MONTERO*‡ and Rhian M. TOUYZ*

*Kidney Research Centre, Ottawa Hospital Research Institute, Ottawa, ON, Canada K1H 8M5, †Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo 05508-900, Brazil, and ‡Department of Physiology and Pharmacology, University of Salamanca, Salamanca 37007, Spain

ABSTRACT

The Noxes (NADPH oxidases) are a family of ROS (reactive oxygen species)-generating enzymes. Of the seven family members, four have been identified as important sources of ROS in the vasculature: Nox1, Nox2, Nox4 and Nox5. Although Nox isoforms can be influenced by the same stimulus and co-localize in cellular compartments, their tissue distribution, subcellular regulation, requirement for cofactors and NADPH oxidase subunits and ability to generate specific ROS differ, which may help to understand the multiplicity of biological functions of these oxidases. Nox4 and Nox5 are the newest isoforms identified in the vasculature. Nox4 is the major isoform expressed in renal cells and appear to produce primarily H2O2. The Nox5 isoform produces ROS in response to increased levels of intracellular Ca2+ and does not require the other NADPH oxidase subunits for its activation. The present review focuses on these unique Noxes, Nox4 and Nox5, and provides novel concepts related to the regulation and interaction in the vasculature, and discusses new potential roles for these isoforms in vascular biology.

INTRODUCTION

ROS (reactive oxygen species) are produced in all layers of the vascular wall by all vascular cell types, including endothelial, smooth muscle and adventitial cells, and by perivascular adipocytes. Among the many enzyme systems responsible for vascular ROS production, including xanthine oxidase, uncoupled eNOS (endothelial NO synthase) and mitochondria, Nox (NADPH oxidase) appears to be particularly important. Nox1, Nox2, Nox4 and Nox5 are the newest isoforms identified in the vasculature. Nox4 is the major isoform expressed in renal cells and appear to produce primarily H2O2. The Nox5 isoform produces ROS in response to increased levels of intracellular Ca2+ and does not require the other NADPH oxidase subunits for its activation. The catalytic subunit is gp91phox, also known as Nox2, of which seven isoforms (Nox1–Nox7) have been identified [1]. In non-phagocytic cells, Noxes produce relatively small amounts of ROS, which act as second messengers in signalling pathways. Nox1, Nox2, Nox4 and Nox5 using NADPH as an electron donor and as such generate ROS. The prototype NADPH oxidase found in phagocytic cells, and critically involved in innate immunity by generating high concentrations of ROS, possesses cytosolic (p47phox, p67phox or homologues) and membrane-bound (gp91phox and p22phox) subunits that upon activation form a functional enzyme complex. The catalytic subunit is gp91phox, also known as Nox2, of which seven isoforms (Nox1–Nox7) have been identified [1]. In non-phagocytic cells, Noxes produce relatively small amounts of ROS, which act as second messengers in signalling pathways. Nox1, Nox2, Nox4 and Nox5

Key words: endothelium, hydrogen peroxide, NADPH oxidase (Nox), reactive oxygen species, superoxide, vascular smooth muscle cell.

Abbreviations: AngII, angiotensin II; CREB, cAMP-response-element-binding protein; eNOS, endothelial NO synthase; EPC, endothelial progenitor cell; ER, endoplasmic reticulum; ET-1, endothelin-1; JAK, Janus kinase; MP, microparticle; Nox, NADPH oxidase; PDGF, platelet-derived growth factor; PKC, protein kinase C; Poldip2, polymerase (DNA-directed) δ-interacting protein 2; REFBID, regulatory EF-hand-binding domain; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; TGF-β, transforming growth factor-β; Tks5, tyrosine kinase substrate with five Src homology 3 domains; TNF-α, tumour necrosis factor-α.

Correspondence: Dr Augusto C. Montezano (email amonteza@uottawa.ca).
are expressed in vascular tissue and are probably the major sources of ROS in the cardiovascular system [1,2]. Nox-derived ROS influence vascular function by modulating constriction/dilation, vascular cell growth, apoptosis, senescence and survival, inflammatory responses, fibrosis, migration and calcification [1,2].

Although there has been enormous progress in the biochemical, chemical and structural characterization of Nox isoforms, the physiological role of Noxes still remains largely unknown, and the exact function in pathological processes awaits clarification. This paucity of information relates, in large part, to the complexity of Nox biology, because Noxes are differentially regulated, are distributed in a cell- and tissue-specific manner, have diverse intracellular localizations and generate different ROS. There is now a concerted effort to better understand the biology of Noxes as highlighted in some excellent recent reviews [1–5].

The present review provides new insights into the Nox isoforms Nox4 and Nox5 in the vasculature. We discuss mechanisms of regulation and highlight some of the key processes involved in activation of Nox4 and Nox5, with particular emphasis on those that are unique to these isoforms. We also provide novel concepts related to the (patho)physiological role of Nox4 and Nox5 in the vasculature and discuss possibilities that cross-talk between Nox4 and Nox5 may be important in vascular cell function.

**NOX4**

Nox4 was initially characterized as a renal gp91 homologue thought to play a role in oxygen sensing and erythropoietin production [6,7]. At first, expression was believed to be specific to the kidney and, indeed, it was initially referred to as Renox by Leto and co-workers [6]. However, there are now reports of Nox4 expression in numerous cell types, including osteoclasts [8], fibroblasts [9], (pre)adipocytes [10,11], keratinocytes [12], neurons [13,14], vascular smooth muscle cells [15,16] and endothelial cells [17–19]. In the vasculature, Nox4 is expressed in adventitial fibroblasts [20] and, on the basis of gene expression studies, appears to be the major isoform expressed in endothelial cells [18,21] and vascular smooth muscle cells [15,22].

Nox4 was originally thought to share structural and functional homology with gp91phox; however, it has since became clear that Nox4 is somewhat distinct in structure from other Nox homologues. In fact, Nox4 shares only 39 % homology with Nox2 and 35 % homology with Nox1 [7]. Additionally, Martyn et al. [23] have reported that, unlike the prototypical gp91phox, Nox4 does not require cytosolic subunits, and is capable of activation in the absence of p47phox/NOXO1 or p67phox/NOXA1. Nox4 does appear to require p22phox as a regulatory subunit, since the expression of Nox4 in a cell line lacking p22phox does not result in ROS generation [23]. There are conflicting reports on the role of Rac1 in Nox4 activity: whereas Nox4 may require Rac1 for activity in vascular smooth muscle cells [24], Nox4 maintains activity when transfected into cells lacking Rac1 [23], and Rac1 inhibition does not appear to block Nox4 activity in endothelial cells [25]. One possible explanation that has been suggested is that Rac1 indirectly mediates ROS production by Nox4 [26].

On the basis of the lack of requirement for cytosolic subunits, Nox4 has traditionally been described as a constitutively active Nox isoform. Regulation of Nox4-derived ROS production was therefore thought to be achieved solely through changes in Nox4 expression levels [27]. However, recent work by Lyle et al. [28] has identified Poldip2 [polymerase (DNA-directed) δ-interacting protein 2] as a putative regulator of Nox4 activity in vascular smooth muscle cells, where it increases Nox4 activity 3-fold [28]. Although Poldip2 expression has been reported in endothelial cells [29], an interaction with Nox4 has not yet been identified. As yet, the mechanisms regulating Poldip2 expression are not known and it is unclear how Poldip2, or other as yet undiscovered regulators of Nox4, may mediate the induction of Nox4 activity. Another regulatory protein that has recently been identified for Nox4 activation is Tks5 (tyrosine kinase substrate with five Src homology 3 domains). Tks5 appears to be particularly important in Nox4-derived ROS generation associated with invadopodia formation [30].

H2O2 appears to be the primary product derived from Nox4-based NADPH oxidase [23,31,32]. The biochemistry underlying this is unclear, but may be due to the rapid dismutation of superoxide to H2O2 following release from Nox4. However, a study by Dikalov et al. [32] could not attribute any measurable superoxide production to Nox4. The authors propose that the dissociation of superoxide from Nox4 is slower than from other Nox isoforms, allowing for the dismutation of superoxide prior to its release from the enzyme. Regardless of when and where dismutation takes place, the ultimate biologically reactive product of Nox4 is generally agreed to be H2O2.

In heterologously expressing cells [23], endothelial cells [18] and vascular smooth muscle cells [33], Nox4 has a strong immunofluorescence in the perinuclear space, suggesting localization to the ER (endoplasmic reticulum). It is possible that this localization is simply an accumulation of Nox4 at its site of synthesis/processing. However, an alternative explanation is that Nox4 plays a role in the ER stress response [33–35]. Pedruzzi et al. [33] reported that ER stress by 7-ketocholesterol in vascular smooth muscle cells is prevented by Nox4 silencing. Nox4 also appears to localize to the nucleus in both vascular smooth muscle cells and endothelial cells and in the mitochondria in vascular...
smooth muscle cells [33,35,36]. Recently, nuclear ROS production by Nox4 has been demonstrated in a cancerous haemangioendothelioma endothelial cell line and implicated in oxidative DNA damage [31]. As yet, it is not known whether a similar pathway exists in healthy endothelial cells or vascular smooth muscle cells. Alternatively, nuclear Nox4/Poldip2 signalling could function in DNA repair [28,37]. Further investigations are required to clarify the role of nuclear Nox4 signalling. Finally, a number of studies have demonstrated localization of Nox4 to stress fibres and to focal adhesions in vascular smooth muscle cells, where it plays a role in vascular smooth muscle cell differentiation and cell migration [15,16,28]. This localization appears to require Poldip2, as silencing of Poldip2 led to a loss of Nox4 localization to stress fibres [28].

Several stimuli are known to increase Nox4 expression in the vasculature, including physiological shear stress [38], ER stress [33], mitochondrial dysfunction [39], atherosclerosis [20,40], arterial injury [41] and hypoxia [42]. Both TGF-β1 (transforming growth factor-β1) and TNF-α (tumour necrosis factor-α) are known to induce Nox4 expression in vascular smooth muscle cells and endothelial cells [42–45]. In fact, it is possible that TNF-α and TGF-β mediate Nox4 induction by many of the above physiological stimuli. Accordingly, TGF-β has been reported to mediate the effects of hypoxia on Nox4 expression [42]. The molecular mechanisms of Nox4 induction may include AP-1 (activator protein-1) [44], JAK (Janus kinase)/STAT (signal transducer and activator of transcription) [46], E2F [47], PEDF (pigment epithelium-derived factor) [48] and PKC (protein kinase C) α [49].

Down-regulation of Nox4 expression in the vasculature has been reported following pathophysiological shear stress [38,50], and exposure to ω-3 fatty acids [51]. Molecular mechanisms for Nox4 inhibition include PKCe [49] and PPAR-γ (peroxisome-proliferator-activated receptor γ) ligands [52]. More recently, angioptietin 2 has been shown to inhibit Nox4 expression in endothelial cells [53], although it is unclear whether a similar pathway exists in non-cancerous endothelial cells.

There is contradictory information with regards to the effects of AngII (angiotensin II) on Nox4 expression in the vasculature. Some studies have shown that AngII administration decreases Nox4 expression in vascular smooth muscle cells [22,32]. On the other hand, vascular smooth muscle cells have been reported to be unresponsive to AngII [39], and still others have reported increased Nox4 expression following AngII administration in vascular smooth muscle cells, thoracic aorta and endothelial cells [17,48,51,54,55]. Further investigation is needed to reconcile these seemingly conflicting observations.

Nox4 has been shown to activate endothelial cells [56], and to cause apoptotic [25] and necrotic [57] cell death following TNF-α treatment and LPS (lipopolysaccharide) treatment respectively. Additionally, Nox4 appears to play a role in endothelial cell migration, since silencing decreases and overexpression increases cell migration [58]. A recent report by Lener et al. [59] found that Nox4 contributes to endothelial cell senescence via oxidative DNA damage. Although the subcellular location of ROS production was not examined in these cells, it is possible that the nuclear localization of Nox4 facilitated this DNA damage. The belief that Nox4 contributes to endothelial cell senescence is supported further by a recent study that found Nox4-derived oxidative stress led to endothelial cell arrest in S-phase [60]. Thus there is considerable evidence to suggest that Nox4 contributes to endothelial cell death and senescence. At the same time, Xu et al. [49] found that silencing of Nox4 blocked VEGF (vascular endothelial growth factor)-dependent endothelial cell proliferation in vitro. Similarly, Datla et al. [58] found that Nox4 overexpression promoted, and Nox4 silencing or expression of a dominant-negative form of Nox4 impaired, endothelial cell proliferation. The latter study also found that Nox4 overexpression inhibited endothelial cell apoptosis.

Similar to endothelial cells, there are conflicting reports on the role of Nox4 in vascular smooth muscle cells. Some early studies found a correlation between Nox4 expression and markers of vascular smooth muscle cell differentiation [20,41]. These observations were supported further by the observation that Nox4 is required for vascular smooth muscle cell differentiation and maintenance of quiescence, possibly through the regulation of serum-response factors [15]. At the same time, however, Nox4 also appears to be required for hyperplasia stimulated by hypoxia and TGF-β, as Nox4 silencing reduces proliferation of pulmonary vascular smooth muscle cells [42,61]. Nox4 may also contribute to vascular smooth muscle cell hypertrophy [61], although others have attributed this effect to Nox1 [62]. Finally, Nox4 appears to be critical for vascular smooth muscle cell migration through regulation of focal adhesions [28]. Interestingly, either knockdown or overexpression of Nox4 impairs vascular smooth muscle cell migration in vitro, suggesting that focal adhesion turnover, both formation and dissolution, are regulated by Nox4 and are critical for vascular smooth muscle cell migration.

The physiological consequences of Nox4 activation in the vasculature are unclear and have been reported to be both inhibitory and stimulatory. On the one hand, several lines of evidence support a beneficial role in the vasculature involving pro-angiogenic anti-apoptotic effects, and a maintenance of smooth muscle differentiation. Moreover, Nox4 is up-regulated by physiological shear stress and down-regulated by pathophysiological shear stress, suggesting further a beneficial role. On the other hand there is ample evidence to support a deleterious role, including the promotion of
endothelial cell senescence, apoptosis, vascular smooth muscle cell migration and hypertrophy. These conflicting results are complicated further by the fact that a Nox4-deficient mouse has yet to be described in the literature. Future studies focusing on tissue-specific Nox4 mutants and more selective inhibitors will allow for clarification of these discrepancies and a more thorough understanding of the role of Nox4 in vascular (patho)physiology.

**NOX5**

Nox5 is the newest member of the NADPH oxidase family to be discovered and characterized. Nox5 was first reported in studies where an mRNA transcript of a novel protein was found in spleen, testis and kidney. This new protein had a significant homology with Nox2 and Nox1 [63,64]. Recent evidence indicates that vascular cells also possess Nox5, of which four splice variants Nox5α, Nox5β, Nox5γ and Nox5δ have been identified [65,66]. Endothelial cells and vascular smooth muscle cells also express the truncated form of Nox5, called Nox5-S. The Nox5 gene is present in humans [63,64], monkeys, cows, dogs, zebrafish and sea urchins. Interestingly, Nox5 is not found in rodents [67,68], making the study of this isoform challenging since mouse or rat models cannot be easily used to study Nox5. Unlike other vascular Noxes, Nox5 possesses an N-terminal calmodulin-like domain with four binding sites for Ca$^{2+}$ (EF hands). Moreover, Nox5 does not require the other NADPH oxidase subunits for its activation [69]. The C-terminal domain possesses binding sites for FAD and NADPH. Nox5 also has a polybasic region that localizes the enzyme to the plasma membrane [63,70]. The splice variant Nox5-S lacks the entire EF-hand region.

Nox5 generates superoxide anions in response to increases in levels of intracellular Ca$^{2+}$. Binding of Ca$^{2+}$ to the EF hand induces a marked conformational change allowing the interaction with a hydrophobic domain on the C-terminal domain [69]. Recently, this hydrophobic domain was characterized in a study by Tirone et al. [71], where the EF hands, once activated by Ca$^{2+}$, bind to two specific domains: the REFBD (regulatory EF-hand-binding domain) and the PhosR (phosphorylation region). The interaction with the EF hands relieves autoinhibitory mechanisms on REFBD [71]. As Nox5 activation is dependent on Ca$^{2+}$, it was initially believed that any stimulus that induces an increase in intracellular levels of Ca$^{2+}$ could induce ROS generation through Nox5. In a cell-free system, although Nox5 activation was still dependent on Ca$^{2+}$, the levels required for a maximum activation of the enzyme were high and unlikely to be achieved in cells [63]. This observation led to later studies demonstrating phosphorylation sites on Nox5 that increase the sensitivity of the enzyme for Ca$^{2+}$, even at resting levels of intracellular Ca$^{2+}$ [72].

Nox5 phosphorylation is regulated in a PKC-dependent pathway [73]. A separate mechanism to increase Nox5 sensitivity to Ca$^{2+}$ is the binding of calcium-bound calmodulin on the calmodulin-binding site of the C-terminal domain of Nox5. The calmodulin-binding site in the C-terminus of a NADPH oxidase is not new or specific for Nox5 since it is also found on Nox4 [74]. Nox5 is also activated by c-Abl (a proto-oncogenic tyrosine kinase) in a Ca$^{2+}$- and H$_2$O$_2$-dependent manner [75]. Recent studies indicate that intracellular Mg$^{2+}$ also modulate Nox5 activity by binding to the EF hands. The biological importance is still unclear, but it is suggested that Mg$^{2+}$ may bind to all four EF hands, limiting the association and dissociation of Ca$^{2+}$ ions [76].

The functional significance of vascular Nox5 is unknown, although it has been implicated in endothelial cell proliferation and angiogenesis [65], in PDGF (platelet-derived growth factor)-induced proliferation of vascular smooth muscle cells [66] and in oxidative damage in atherosclerosis [77]. Vascular Nox5 is activated by thrombin, PDGF and ionomycin through PKC and CREB (cAMP-response-element-binding protein) [65,66,73]. The intracellular location of Nox5 can have a profound impact on the range of molecules that are targeted by the enzyme. Nox5 is found in the perinuclear area in human endothelial cells which co-localizes with ER [65,78]. Nox5 generates both superoxide anions and H$_2$O$_2$ [79]. H$_2$O$_2$ is membrane-permeant, whereas superoxide is not, which would suggest that only Nox5-derived H$_2$O$_2$ would be detectable in the extracellular milieu. In human vascular smooth muscle cells, Nox5 is also found in the plasma membrane (A.C. Montezano and R.M. Touyz, unpublished work) as well as in other cell types [78], and it is related to a polybasic domain that binds to phosphatidylinositol 4,5-biphosphate, which is a phospholipid found in the plasma membrane [70].

Considering the unique Ca$^{2+}$ and calmodulin-like domain of Nox5, it is possible that agonists signalling through increased intracellular Ca$^{2+}$ levels, as demonstrated previously for AngII and ET-1 (endothelin-1) [80], regulate Nox5 activity/expression. The molecular mechanisms controlling Nox5 expression are not fully understood, but CREB activation has been implicated [77]. AngII and ET-1, but not aldosterone, increased Nox5 gene and protein expression through transcriptional and translational mechanisms, where CREB activation was involved. Furthermore, these Nox5 effects were related to an increase in intracellular Ca$^{2+}$ levels, in part through L-type Ca$^{2+}$ channels, and calmodulin activation in human microvascular endothelial cells [78]. AngII- and ET-1-induced ROS generation was dependent on Nox5 activation, since knockdown of the enzyme blocked agonist-stimulated ROS production. In Nox5-down-regulated endothelial cells, ERK1/2 (extracellular-regulated kinase 1/2) activation was also decreased after stimulation with AngII and
Endothelial-cell-derived MPs are released by stimuli that cause apoptosis or endothelial cell injury. Once released into the blood stream, MPs may be carrying Nox4 and/or Nox5, leading to ROS formation and release to endothelial cells in the vasculature. MP-derived ROS then influence expression and/or activity of other Nox homologues (Nox1, Nox2, Nox4 and Nox5) in the endothelium, which induce the activation of redox-sensitive signalling, leading to endothelial cell senescence, apoptosis/growth, fibrosis and inflammation. During endothelial damage (e.g. hypertension and atherosclerosis), vascular smooth muscle cells are exposed to endothelial-derived MPs, which in turn activate vascular Nox homologues and redox-sensitive signalling, leading to vascular smooth muscle cell constriction/dilation, hypertrophy, fibrosis and inflammation, all features of vascular remodelling.

ET-1. However, Nox5 appears to be critical in growth and inflammatory responses by AngII and not for ET-1 [78].

Nox5 is expressed in vascular smooth muscle cells from human coronary arteries and aorta [66,76], and in endothelial cells from human microvessels [78]. Nox5 is also involved in PDGF-induced ROS generation, leading to JAK/STAT pathway activation and cell proliferation [66]. In whole vessels, Nox5 was also shown to induce eNOS activity, an effect associated with increased binding of eNOS with HSP90 (heat-shock protein 90). Despite this, Nox5 reduced NO bioavailability, impaired endothelium-dependent relaxation and increased phenylephrine-induced contraction [81]. Recently, Guzik et al. [77] demonstrated that, in coronary arteries from patients with coronary artery disease, Nox5 protein and mRNA levels are increased and Ca2+-dependent NADPH-driven production of ROS in vascular membranes is augmented. In that study, they also found that Nox5 localized in the endothelium in the early-stage lesions and in vascular smooth muscle cells in the advanced coronary lesions, linking Nox5 and ROS generation in atherosclerosis [77]. In vascular smooth muscle cells from hypertensive subjects, Nox5 appears to be up-regulated compared with cells from normotensive subjects, and is regulated by AngII, an effect also dependent on Ca2+ and calmodulin activation (A.C. Montezano and R.M. Touyz, unpublished work). These observations are important and suggest that Nox5 may be an important source of ROS in hypertension.

Dysregulation of these processes, due to augmented signalling by PDGF, thrombin, AngII and ET-1, may lead to Ca2+ overload and Nox5-derived oxidative stress,
which could contribute to endothelial dysfunction and vascular disease. Mechanisms whereby Nox5 expression and function are regulated are still far from being elucidated, since Ca$^{2+}$ is not the sole activator of the enzyme.

Although the physiological significance of Nox4 and Nox5 remain elusive, there is evidence that these isoforms may be important in pathological conditions, since their expression and activity are increased in disease states where vascular remodelling is important, such as in hypertension, diabetes and atherosclerosis (for reviews, see [5,22,82]).

ROLE OF NOX4 AND NOX5 IN VASCULAR BIOLOGY: NEW CONCEPTS

A new paradigm for Noxes relates to ROS production in MPs (microparticles). MPs are nano-fragments of membranes that originate from activated or apoptotic cells, are found in the bloodstream and are approx. 0.1–1.0 μm in size [83]. These MPs have traditionally been thought of as biomarkers of vascular disease; however, there is emerging evidence that they may themselves be biologically active, modulating such processes as vasorelaxation [84] and inflammation [85]. In 2004, Brodsky et al. [84] reported that endothelial MPs produce ROS and contain the Nox subunit p22$^{phox}$. Although the authors did not investigate the presence of other Nox components, a subsequent study by Mostefai et al. [86] found Nox1 and Nox4 in lymphocytic MPs, and results from our laboratory has found Nox4 in endothelial MPs (D. Burger and R.M. Touyz, unpublished work). Thus it is possible that these tiny fragments of cell membrane are actually redox-active biological effectors. Nox4 might be uniquely suited among Nox isoforms to produce ROS in MPs, since this isoform does not require the presence of cytosolic subunits for its enzymatic activity (Figure 1).

There is also growing evidence to suggest a role for Nox4 in vascular aging. This provides a mechanism for the long-postulated theory that free radicals accelerate aging [87,88]. Age-associated changes in the vasculature include smooth muscle polyploidy, endothelial dysfunction, endothelial cell senescence and decreased EPC (endothelial progenitor cell) levels [89–91]. Nox4 appears to promote endothelial cell senescence [59,93]. Additionally, McCrann et al. [87] recently found that Nox4 expression increases with age in rat aortas and isolated vascular smooth muscle cells. This increase is associated with polyploidy of vascular smooth muscle cells and overexpression of Nox4 increased vascular smooth muscle cell polyploidy, establishing a causal relationship between Nox4 and polyploidy. Moreover, Nox4 has been implicated in EPC apoptosis [94] and in endothelial dysfunction [95]. Thus Nox4 may be intimately involved in redox processes associated with...
vascular aging. Nox4 is also implicated in adipocyte differentiation, which may be important in perivascular adipose tissue biology [96] (Figures 2 and 3).

All cells express multiple Nox isoforms. Studies have shown that Nox isoforms can be similarly regulated by the same stimulus, co-localize in the same cell compartment and yet have different functional effects [97]. Whereas Nox1 is required for hypertrophy and proliferation, Nox4 is essential for cell differentiation and Nox5, because of its Ca\(^{2+}\)-dependent regulation, may be involved in vascular cell contraction and growth [98–103]. New findings indicate that Nox isoforms may actually dimerize, as suggested for Nox2 and Nox4 [104]. We suggest that Nox4 and Nox5 may also interact, since Nox5 down-regulation in human endothelial cells, which express Nox4 and Nox5, resulted in abrogation of NADPH-oxidase-derived ROS production, even though Nox5 was not completely knocked down [78]. These responses were not compensated for by increased Nox4 or Nox2 expression. Moreover, unpublished observations have shown that NADPH oxidase-derived ROS was also blocked by p22 knockdown, but not by Rac-1 inhibition (A.C. Montezano and R.M. Touyz, unpublished work). Reasons for these effects are complex, but may be related, at least in part, to the fact that Nox5 down-regulation is associated with other subunits and/or Nox isoforms, including Nox4 (Figure 2).

Finally, even though there is growing information regarding Nox4 regulation, signalling and biological functions, it is still unclear whether Nox4 plays a protective or deleterious role in vascular diseases, and it is unknown how the fine-tuning for its activity is lost during disease states. As for Nox5, there is a paucity of information on the regulation, signalling and function of this Nox family member. We know that vascular cells possess multiple Nox isoforms, but which isoform represents the major enzymatic source of ROS in the vasculature and plays a predominant role in vascular diseases is still unclear. Regardless, what is clear is that ROS, once generated by activated Noxes,
appear to regulate their own production through a feed-forward system, thereby contributing to increased and sustained oxidative stress, implicated in vascular damage. Additional studies are still required to better understand these novel isoforms (Nox4 and Nox5) and the interactions between other Nox family members and other NADPH oxidase subunits including p22phox, p47phox, p67phox, NOXO1 and NOXA1 leading to a pro-oxidative positive-feedback mechanism important to vascular diseases.

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