ANTIOXIDANTS & REDOX SIGNALING
Volume 15, Number 6, 2011

DOI: 10.1089/ars.2010.3614

FORUM REVIEW ARTICLE

Redox Regulation of Protein Kinases as a Modulator of Vascular Function

Greg A. Knock and Jeremy P.T. Ward

Abstract

Reactive oxygen species (ROS) are continuously generated in vascular tissues by various oxidoreductase enzymes. They contribute to normal cell signaling, and modulate vascular smooth muscle tone and endothelial permeability in response to physiological agonists and to various cellular stresses and environmental factors, such as hypoxia. While concentrations of ROS are normally tightly controlled by cellular redox buffer systems, if produced in excess they may contribute to vascular disease. Protein kinases are essential components of most cell signaling pathways, including those involving ROS. The functioning of several members of this highly diverse group of enzymes, which include receptor and nonreceptor tyrosine kinases, protein kinase C, mitogen-activated kinases, and Rho-kinase, are modified by ROS, either through direct oxidative modification or indirectly through modification of associated proteins such as tyrosine phosphatases and monomeric G proteins. In this review, we discuss the molecular mechanisms of redox modification of these proteins, the downstream pathways affected, the often complex interaction between major kinase pathways, and feedback to ROS production itself. We also discuss complicating factors such as differential actions of superoxide anion and hydrogen peroxide, questions concerning concentration dependence, and the significance of signaling microdomains. *Antioxid. Redox Signal.* 15, 1531–1547.

Introduction

Reactive oxygen species (ROS) are diffusible, short-lived molecules that can produce functional modifications within biological systems through the reversible oxidation of a diverse range of molecules such as membrane lipids, protein kinases, ion channels, and transcription factors (47). They are generated in cells by oxidoreductase enzymes such as NADPH oxidases, xanthine oxidase, cyclooxygenase, lipoxygenase, nitric oxide (NO) synthase, hemoxygenase, peroxidases, and within the mitochondrial electron transport chain (28, 46, 92). ROS act as important second messenger molecules, and in vascular tissues may mediate responses such as gene expression, growth, survival/apoptosis, migration, adhesion, inflammation (4, 47, 124, 126), as well as modulating smooth muscle contractility and endothelial permeability (see below). Protein kinases, a highly diverse group of phosphotransferase enzymes that alter the function of target proteins by catalyzing phosphorylation of tyrosine, threonine, and/or serine residues, are key components of cell signaling. A wide variety of protein kinases are implicated in smooth muscle constriction, including receptor tyrosine kinases (*e.g.*, epidermal growth factor receptor [EGF-R] and platelet-derived growth factor-receptor), the src family of nonreceptor tyrosine kinases (srcFK), protein kinase C, mitogen-activated protein kinases (MAPKs), and Rho-kinase (152), and, as will be seen, many are prime targets for redox regulation.

The principal ROS produced by most oxidoreductases is superoxide ($O_2^{\bullet -}$), from incomplete reduction of molecular oxygen. This is converted to the more stable hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) in the cytosol, mitochondria, and extracellular matrix. H_2O_2 is destroyed by catalase or glutathione peroxidise (by reduction to H_2O), but can be converted to hydroxyl radical ($OH^{\bullet}$) by the Fenton reaction (catalyzed by Fe^{2+}). Superoxide and H_2O_2 are thought to be of greatest physiological relevance, $OH^{\bullet}$ being considered too reactive to have meaningful signaling properties. Superoxide also reacts with NO, forming peroxynitrite ($ONOO^-$), and growing evidence suggests that this can oxidatively modify proteins through nitration or S-nitrosylation (80), although it has been argued that it is too indiscriminate to be of physiological relevance aside from triggering cell damage, apoptosis, and necrosis (57).

The redox state of vascular tissues is normally tightly controlled. A balance between oxidative stimuli, antioxidant enzymes, and the glutathione redox buffer normally keeps the interior of the cell in a reduced condition. When this balance is shifted in favor of oxidation (oxidative stress) (35), it may

Division of Asthma, Allergy, and Lung Biology, King's College London, London, United Kingdom.

result in dysregulation of signaling pathways in smooth muscle and endothelium, thus contributing to various pathologies, including hypertension (28, 63, 127, 145), diabetes (34), hypoxia/re-perfusion injury (62, 161), and atherosclerosis (30, 102), and may ultimately result in cell damage and apoptosis (57) (Fig. 1). However, the responses to several physiological stimuli in vascular cells are believed to be mediated, at least in part, *via* enhanced ROS production. These include activation of G-protein-coupled receptors by angiotensin II (Ang II), endothelin-1 (ET-1), and thrombin (28, 46, 102, 157); mechanical forces and shear stress (30, 98, 145); and even depolarization and temperature (11, 14).

While most of the above involve activation of NADPH oxidase, there is evidence that ROS derived from mitochondria play an important signaling role in certain circumstances. A specific example of the latter is hypoxic pulmonary vasoconstriction (HPV), where it has been proposed that the initial stimulus is an hypoxia-induced elevation of mitochondrial ROS production (112, 154–156), although, in contrast, others have suggested that HPV is initiated by falling mitochondrial ROS production and a more reduced redox state (93, 158) [see also (7, 151)]. An hypoxia-induced increase in mitochondrial ROS production has been shown to activate NADPH oxide *via* protein kinase C (PKC) in pulmonary artery smooth muscle cells (PASMCs) (112). In addition, mitochondrial ROS may contribute to chronic hypoxia-associated pulmonary hypertension (14, 58). Importantly, physiological responses to many of the above-described stimuli and associated changes in protein kinase activity are commonly inhibited by antioxidants, strongly suggesting that ROS act as signaling mediators between the stimulus and downstream signaling pathways (1, 14, 58, 102, 110, 145).

For example, SOD generally relaxes agonist or pressure-induced arterial constriction (1, 145), suggesting a role for superoxide in smooth muscle constriction. This is consistent with the general constrictor role for exogenously applied superoxide derived either from xanthine/xanthine oxidase or quinones such as LY83583 and menadione (9, 50, 69). Only part of this constriction is endothelium-dependent through scavenging of NO (9, 39, 69, 98, 113). Vascular beds where externally applied ROS cause constriction include aorta and vena cava (8, 142), pulmonary artery (60, 97, 103, 104, 108, 128, 130), coronary artery (98), cerebral artery (165), human umbilical artery (101), and mesenteric artery (8, 39, 83). Notably, ROS-mediated vasoconstriction is inhibited by nonspecific tyrosine kinase inhibitors (*e.g.*, genistein) (8, 165), and specific inhibitors of srcFK (60, 69, 142), p38, or extracellular signal-regulated kinase (ERK) MAPKs (8, 83, 97, 142, 165), Rho-kinase (8, 59, 69, 142), and PKC (108, 128, 165). However, exogenous ROS are also reported to cause relaxation, depending on the vascular bed and experimental conditions, particularly the nature of any preconstriction (39, 69, 83, 122). Notably, H_2O_2 causes relaxation of arteries preconstricted with agonists such as phenylephrine or prostaglandin $F_{2\alpha}$ (39, 83, 86), but not with raised $[K^+]$ (8, 83). This suggests that H_2O_2 may cause hyperpolarization, perhaps through direct redox-regulation of Kv channels (39, 116), although actions *via* protein kinases cannot be ruled out.

From the above it is clear that ROS play an important role in the physiology and pathophysiology of vascular function. In this review, we focus on the interactions between ROS and protein kinase signaling that relate to the control of vascular smooth muscle tone and endothelial permeability, both of which employ similar actin-myosin-based constrictor mechanisms. Several processes have been suggested by which protein kinase activity may be modified by oxidation. Principal among these are selective oxidation and/or nitrosylation of key redox-sensitive cysteine residues either in the kinase itself or in regulatory proteins such as tyrosine phosphatases, which have unusually low ionization pKa of 4–5 compared to the typical pKa of 8.5 of nonreactive cysteines in most other proteins (57, 105, 143). Cysteine oxidation may result in either inhibition or activation, and may or may not cause formation of intra- or intermolecular disulfide bridge formation, depending on the target protein tertiary structure. Additionally, nitration or nitrosylation of tyrosine residues by peroxynitrite may prevent tyrosine phosphorylation (80), but whether this can be as tightly regulated as cysteine oxidation is a matter of dispute (57). Here, we discuss the evidence for direct and indirect redox regulation of key protein kinases relevant to vascular tone and endothelial permeability, how these kinases interact with each other in relation to redox signaling, and

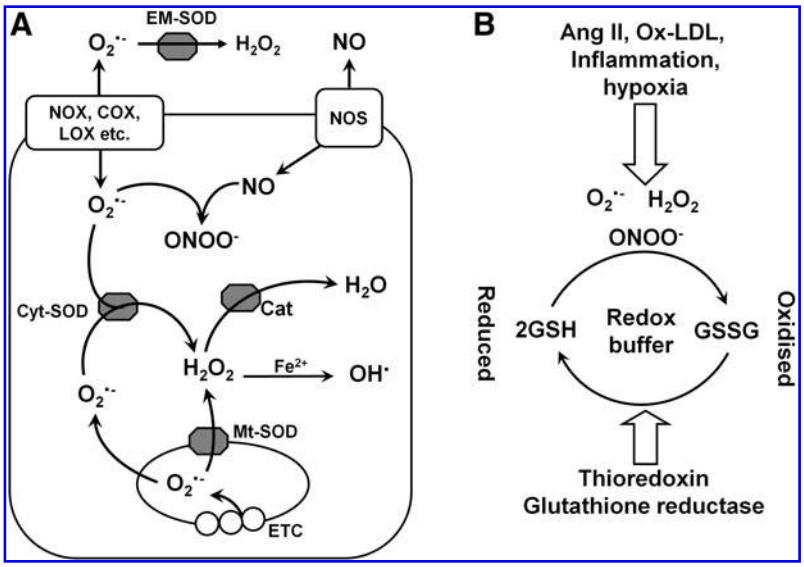

FIG. 1. Sources of reactive oxygen species (ROS) in vascular tissues. (A) Superoxide ($O_2^{\bullet-}$) is generated by membrane-bound enzymes, including NADPH oxidase (NOX), lipoxygenase (LOX), and cyclooxygenase (COX), and in the mitochondria by the electron transport chain (ETC). Superoxide is either converted to hydrogen peroxide (H_2O_2) by mitochondrial, cytoplasmic, or extracellular superoxide dismutase (Mt-SOD, Cyt-SOD, and EM-SOD, respectively), or reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$). H_2O_2 is destroyed by catalase (Cat) or converted to hydroxyl radical ($OH^{\bullet}$) by the Fenton reaction (catalyzed by Fe^{2+}). **(B)** External stimuli such as angiotensin II (Ang II), oxidized low-density lipoprotein (LDL), inflammation, or hypoxia cause excess ROS production. This is balanced by the redox buffer glutathione, which donates electrons through thiol oxidation and dimerization. This process is reversed by thioredoxin and glutathione reductase.

how they may be of relevance to normal and abnormal cell signaling during oxidative stress.

SrcFK and Tyrosine Phosphatases

Tyrosine phosphorylation is essential to early signaling events in response to various stimuli, many of which are associated with increased ROS production. There is strong evidence that tyrosine kinase signaling plays an important role in the control of vascular tone, since tyrosine phosphatase inhibitors generally constrict smooth muscle, whereas tyrosine kinase inhibitors cause relaxation (22, 33, 74, 144). Notably, ROS increase total tyrosine phosphorylation in vascular tissues (33, 36, 74). Interest has particularly focused on srcFK, which are best known for their involvement in integrin activation, cytoskeletal reorganization, and cell spreading, but have also been implicated in signal transduction of constrictor responses to hypoxia (70, 125), stretch (98), Ang II (48, 55, 144, 147), and $PGF_{2\alpha}$ (68). Many of the above appear to be mediated, at least in part, *via* ROS (36, 48, 55, 125, 129, 147), and superoxide-induced constriction in pulmonary artery is suppressed by inhibition of srcFK (69). The pathway(s) through which srcFK mediate constriction are still under investigation, but downstream links to ERK MAPK, p38 MAPK, and the Rho/Rho-kinase pathway, as well as tandem activation with PKC, have been suggested, and these interactions will be discussed in later sections. ROS-activated srcFK also influences the actin cytoskeleton in association with other nonreceptor tyrosine kinases FAK and Pyk-2 (135).

Src may be a target for either direct redox regulation and/or regulation indirectly through redox modification of associated proteins, particularly tyrosine phosphatases. However, understanding of the molecular mechanisms first requires a description of how the folding of the src protein controls activity of its kinase domain. Src protein is composed of several domains, an N-terminal membrane binding domain, an SH3 domain for binding to proline-rich sequences, an SH2 domain for binding phospho-tyrosine, a proline-rich linker region, a catalytic domain containing a kinase activation loop, and a C-terminal regulatory domain. Src exists in two principal conformations, a folded inactive form and an open active form. Src also contains two important phospho-tyrosine residues, one in the kinase activation loop (tyr-418 in humans) and one in the C-terminal regulatory domain (tyr-527 in humans). The inactive form is kept folded by internal binding of the SH2 domain with phosphorylated tyr-527 in the regulatory domain, and this folded configuration is further stabilized by internal binding of the SH3 domain with the proline-rich linker region (118, 138) (Fig. 2A).

Classically, src is activated by destabilization of this internal binding either by de-phosphorylation of tyr-527 or by binding of proteins with phospho-tyrosine residues or proline-rich regions, such as the platelet-derived growth factor-receptor (118, 138). Either way, these processes result in autophosphorylation of tyr-418 in the activation loop, thereby fully activating the kinase (138). However, tyr-418 may also be phosphorylated by other kinases (19), and phosphorylation of tyrosine or serine residues within the SH2, probably by PKC, further destabilizes internal binding (118, 147). Src is subsequently inactivated through either dephosphorylation of tyr-418 or re-phosphorylation of tyr-527 by C-terminal src kinase (CSK) (27, 118).

Many stimuli that generate ROS also activate SrcFK, and a causative link between the two has been suggested by studies that either measured src activity directly *in vitro*, or used tyr-418 phosphorylation as a measure of activity. Such stimuli include laser irradiation (168), hypoxia/reoxygenation (77, 125), cell adhesion, integrin activation and stretch (40, 98), growth factors (76), B-cell receptor activation (133), and agonists such as Ang II and thrombin (48, 55, 117, 129, 147). The source of ROS responsible for srcFK activation in response to these stimuli appears location dependent. For example, src recruited to focal adhesions requires locally recruited lipoxygenase (20, 40), endosomal src is activated by endosomal NADPH oxidase (77), and mitochondrial ROS activate src in response to hypoxia (125), whereas src activation associated with Ang II stimulation, stretch, or lactate-induced constriction are linked with membrane NADPH oxidase activity (48, 97, 98, 129). Notably, src is also activated by exogenous H_2O_2 in various tissues, including vascular smooth muscle, and at concentrations believed to be within the physiological range (5, 88, 117, 120, 168). However, two recent studies suggest that, at least in some cell types, src is inactivated by H_2O_2 (65, 141).

Direct redox regulation of src

SrcFK proteins contain several cysteine residues that are functionally important and potentially confer redox sensitivity to the kinase. Two such residues are cys-245 in the SH2 domain and cys-487 in the kinase domain, which have been shown to confer sensitivity to lipoxygenase-derived ROS at focal adhesions (40). This study showed that integrin-induced src activation was bi-phasic, the first phase involving dephosphorylation of tyr-527 mediated by the trans-membrane protein tyrosine phosphatase (PTP), PTP-α and subsequent tyr-418 autophosphorylation, and the second involving oxidation of cys-245 and cys-487, resulting in yet further activation of the kinase through hyper-phosphorylation of tyr-418. In this model, the second-phase ROS-mediated activation still required prior de-phosphorylation of tyr-527 and was inhibited by antioxidants or substitution of cys-245 and cys-487 with alanine (40) (Fig. 2B). Alternatively, srcFK may also be activated without prior de-phosphorylation of tyr-527 through oxidation of as yet unidentified cysteine residues by either H_2O_2 or NO (5). As this activation was reversed by dithiothreitol, it is likely that disulfide bridge formation was essential for tyr-527-independent src activation. These disulfide bridges were causing clustering of adjacent src molecules and presumably destabilizing intramolecular SH2/SH3 binding (5). Clustering of the kinase in this way may promote *trans*-phosphorylation of tyr-418 by protecting it from dephosphorylation (106).

In contrast to the above, it has been suggested that intramolecular disulfide bridge formation between adjacent src molecules at cys-277 causes inactivation, since in a cell-free system purified src kinase activity was enhanced by dithiothreitol but was unaffected by H_2O_2, though the latter reversed activation by dithiothreitol (65). This suggests that under these experimental conditions the purified kinase is oxidized and thus inhibited in the absence of dithiothreitol. The question remains, however, how these conditions relate to the reduced environment within intact cells.

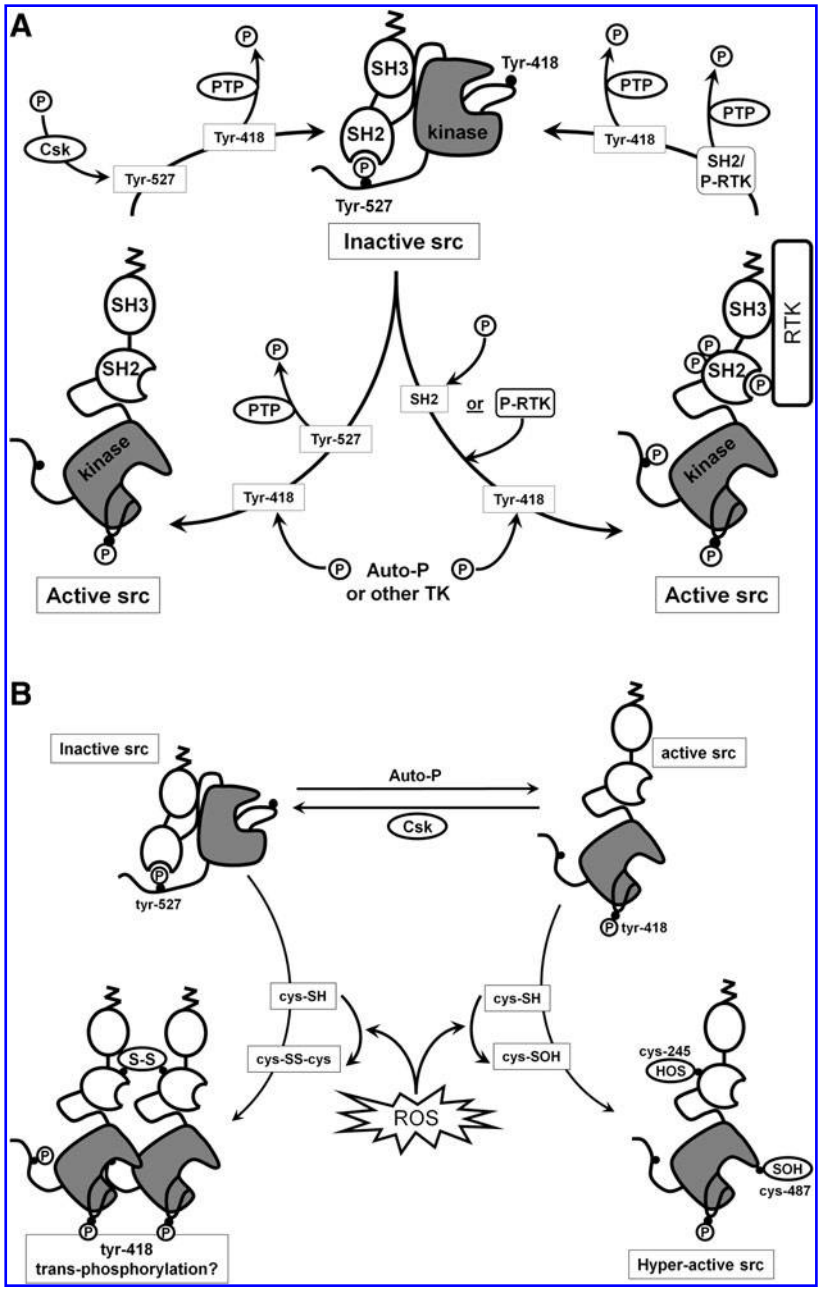

FIG. 2. Structure and direct redox regulation of Src. (A) Src family kinases contain an SH3 domain, a phospho-tyrosine-binding SH2 domain, a kinase domain, and a C-terminal regulatory domain. Inactive src is folded upon itself through internal binding of SH2 with phosphorylated tyr-527. De-phosphorylation of tyr-527 by protein tyrosine phosphatases (PTP), or disruption of SH2 binding by other phosphorylated proteins or SH2 phosphorylation, all promote autophosphorylation of tyr-418, and kinase activation. Src is inactivated either by Csk-dependent phosphoylation of tyr-527, or de-phosphorylation of associated phospho-proteins, followed by tyr-418 de-phosphorylation. **(B)** Two proposed modes of src activation by direct oxidation. Oxidation of cysteine residues in the SH2 of inactive src causes disulfide bridge formation (cys-S-S-cys) and clustering of src (*left*). This promotes trans-phosphorylation of tyr-418. Alternatively, oxidation of cys-245 (-SOH) in the SH2 and of cys-487 in the kinase domain of active src causes hyper-phosphorylation of tyr-418 (*right*).

Indirect redox regulation of src

Exogenous H_2O_2, peroxynitrite, and stimuli that elevate endogenous ROS production usually cause a net increase in cellular tyrosine phosphorylation, indicating a net inhibition of PTP activity relative to tyrosine kinase activity (36, 91). Several studies have demonstrated inhibition of PTPs by H_2O_2 and other cellular oxidants such as oxidized glutathione (GSSG) both *in vitro* and in intact cells, and reversal of these effects by endogenous antioxidants such as thioredoxin and reduced glutathione (GSH) (31, 76, 123). All PTPs contain cysteine residues in their catalytic domains that are essential for catalytic activity. Analysis of the amino acid sequences of these domains within structurally diverse PTPs show that they share a highly conserved signature active site motif that appears to confer redox sensitivity to the active site cysteine residues by dramatically lowering their pKa for ionization, therefore rendering them more sensitive to oxidation than cysteine residues of most other proteins (105, 143). This oxidation is slowly but readily reversible providing it does not proceed beyond sulfenic acid (-S-OH), and may promote disulfide bridge formation, though this is not always necessary for inhibition of catalytic activity (143). As well as opposing the actions of tyrosine kinases on downstream target proteins, PTPs also de-phosphorylate both tyr-527 and tyr-418 of src. The net effect of redox state on tyr-418 phosphorylation and src activity will depend on the relative redox sensitivity of the PTPs that de-phosphorylate the two tyrosine residues, which PTPs are expressed, and the finely tuned spacio-temporal targeting of ROS production to specific PTPs within the various cellular microdomains (133). Presently, we do not have a complete picture of which PTPs de-phosphorylate tyr-

527 and tyr-418. Whereas tyr-527 can be de-phosphorylated by the cytosolic PTP1-B, SHP-1, SHP-2, and trans-membrane PTP-α and CD45, some reports suggest that only the cytosolic PTP-BL and perhaps CD45 can de-phosphorylate src at tyr-418 (75, 106, 118, 137, 141) (Fig. 3A). Oxidative inhibition of PTPs may also contribute to activation of MAPKs by ROS, as discussed in a later section.

Activity of purified Csk, the kinase that inactivates src through phosphorylation of tyr-527, is enhanced by dithiothreitol, probably through reduction of a specific disulfide bridge in its SH2 domain that has been shown to limit its activity (90). This disulfide bond persists in the presence of modest levels of dithiothreitol (25–100 m*M*), so may exist in the generally reducing environment within living cells (90). If so, increased ROS would most likely enhance or preserve integrity of this disulfide bridge and subsequently limit Csk activity, resulting in enhanced src activity. Alternatively, Csk is also known to form an intermolecular disulfide bridge with src, also potentially altering Csk-mediated src inactivation (65) (Fig. 3a). A number of other redox-sensitive processes potentially activate src by destabilizing internal SH2-phosphotyrosine binding. In vascular smooth muscle, Ang II-induced src activation is associated with antioxidant-sensitive phosphorylation of the SH2 domain (147). In addition, in erythrocytes the srcFK lyn is activated by peroxynitrite through nitration of an unidentified accessory protein that displaces phosphorylated tyr-527 from the SH2 domain, thus activating the kinase without the requirement for prior dephosphorylation of that residue (85, 91) (Fig. 3B).

Positive feedback between src activation and ROS production

A number of studies indicate that src is part of a positive feedback loop that results in a boosting of ROS production

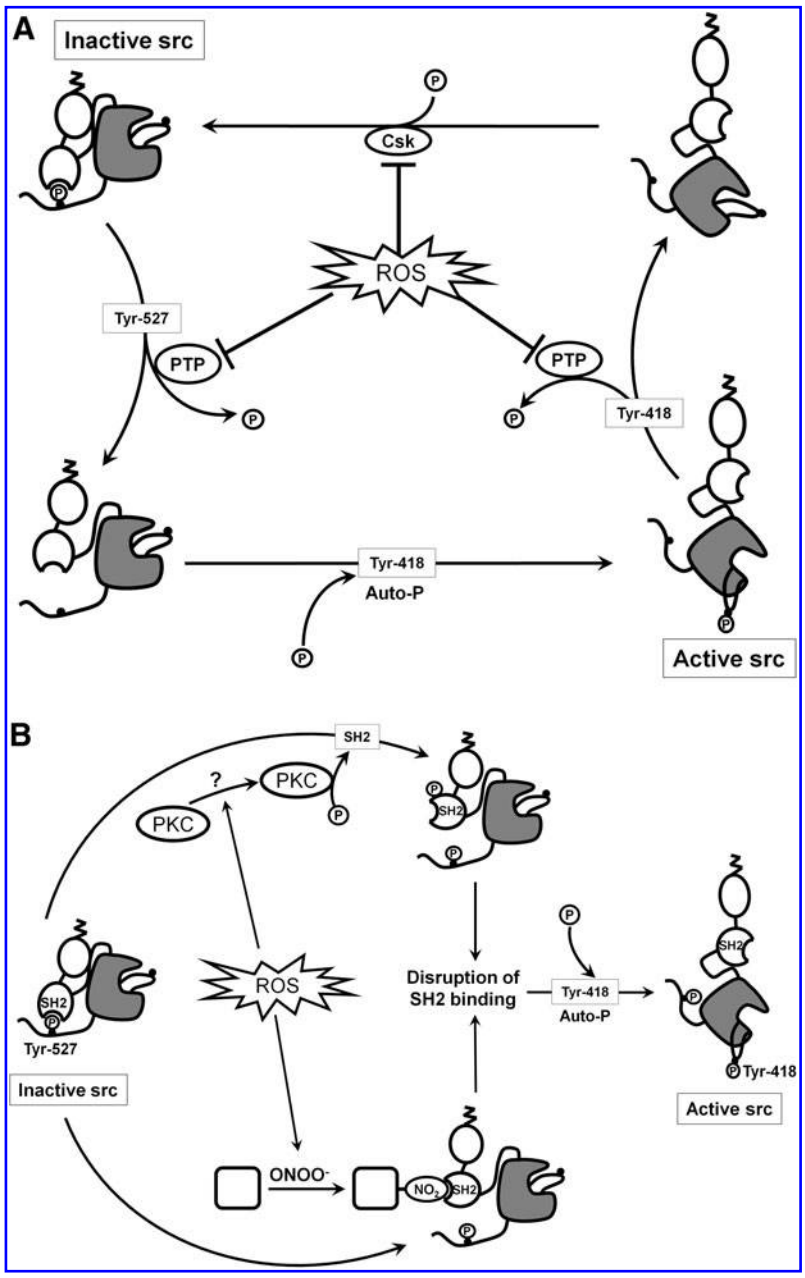

FIG. 3. Indirect redox regulation of src. (A) Inhibition of Csk by cysteine oxidation prevents phosphorylation of src tyr-527, keeping src active for longer (*top*). Inhibition of PTPs that de-phosphorlyate tyr-418 also enhances src activity (*right*). However, inhibition of PTPs that de-phosphorylate tyr-527 prevents activation of src. **(B)** Binding of the SH2 domain to phosphorylated tyr-527 is disrupted by either ROS-directed SH2 domain phosphorylation (*top*), or by an as yet unidentified nitrated protein (*bottom*), resulting in tyr-418 autophosphorylation and activation of src.

from NADPH oxidase through a number of different pathways (23, 55, 129, 133, 144). In human vascular smooth muscle, src is upstream of serine phosphorylation of $p47^{phox}$ in response to Ang II, suggesting an intermediary serine/threonine kinase (144), perhaps PKC (see below), whereas in endothelial cells src apparently mediates tyrosine phosphorylation of $p47^{phox}$ (23). In rat aortic smooth muscle, Ang II causes an initial activation of NADPH oxidase, subsequent ROS-dependent activation of src, which then through transactivation of the EGF-R, activates rac-1, which is required for assembly and activation of NADPH oxidase (129).

Interaction between src and receptor tyrosine kinases

Trans-activation of growth factor receptors is a way of activating the receptor tyrosine kinase in the absence of extracellular agonist. Oxidative bursts induced either by Ang II/src or external stresses such as ultraviolet light or direct application of oxidants such as H_2O_2 and peroxynitrite cause antioxidant-inhibitable and src-dependent tyrosine phosphorylation and activation of receptor tyrosine kinases such as EGF-R in the absence of EGF (107, 147, 165). The phosphorylated receptor then acts as a signaling platform for the stimulation of phospholipase enzymes, production of lipid mediators, and activation of downstream kinases such as PI3-K, Akt, ERK, and PKC (140, 165) (see below), as well as activation of rac-1, feeding back to NADPH oxidase (129).

Protein Kinase C

PKC is a family of cytosolic serine/threonine kinases consisting of 12 isozymes divided into three subclasses: conventional, novel, and atypical. Conventional PKCs require Ca^{2+} and diacylglycerol or phorbol ester for activation, whereas novel isozymes are diacylglycerol-dependent but Ca^{2+}-insensitive; atypical PKCs require neither diacylglycerol nor Ca^{2+}, but are activated by various other lipid mediators (136). Of all the isozymes, three conventional (α, β1/2), two novel (δ, ε), and one atypical (ζ) are reportedly expressed in vascular tissues (81). All PKC isozymes are composed of a C-terminal catalytic domain, a central hinge region and an N-terminal regulatory domain containing an autoinhibitory pseudosubstrate (136). In the regulatory domain several cysteine residues are coordinated with Zn^{2+} and this is required for maintaining the kinase in its folded inactive form. Binding of Ca^{2+} and/or lipid activators triggers release of Zn^{2+}, followed by release of the pseudo-substate, and subsequent unfolding of the kinase and activation of the catalytic domain (66, 72, 136). PKC is the target for multiple tyrosine phosphorylations by various tyrosine kinases, including srcFK (136). These modifications further enhance PKC kinase activity after it is activated by diacylglycerol (78, 119) (Fig. 4). PKC is involved in G-protein-coupled receptor-phospholipase C-mediated smooth muscle constriction, primarily *via* phosphorylation of CPI-17, a myosin accessory protein that inhibits catalytic activity of myosin phosphatase, thus increasing the sensitivity of myosin light-chain kinase to Ca^{2+} (166). Increased PKC activity has also been associated with various acute and chronic vascular stresses such as hypoxia and ischemia reperfusion, and the mechanical strains associated with atherosclerosis and restenosis (164).

PKC is activated by H_2O_2 or peroxynitrite in pulmonary artery smooth muscle and endothelium (15, 16). H_2O_2 also triggers subcellular translocation of various PKC isozymes, including PKC-α in PASMCs (16, 44, 108). H_2O_2-mediated constriction in canine coronary artery and rat pulmonary artery was inhibited by blockers of PKC, in the latter case by the specific inhibitor of conventional PKC Gö6976 (108, 165).

Both PKC and ROS have been implicated in HPV (81, 154–156). Recent work in mice suggests a link between hypoxia, mitochondrial ROS, PKC-ε, and elevation of $[Ca^{2+}]i$. In PASMC, but not in mesenteric artery smooth muscle cell (MASMC), hypoxia triggered rotenone-sensitive mitochondrial ROS production, enhanced PKC-ε activity, increased $[Ca^{2+}]i$, and caused constriction. Although MASMC did not

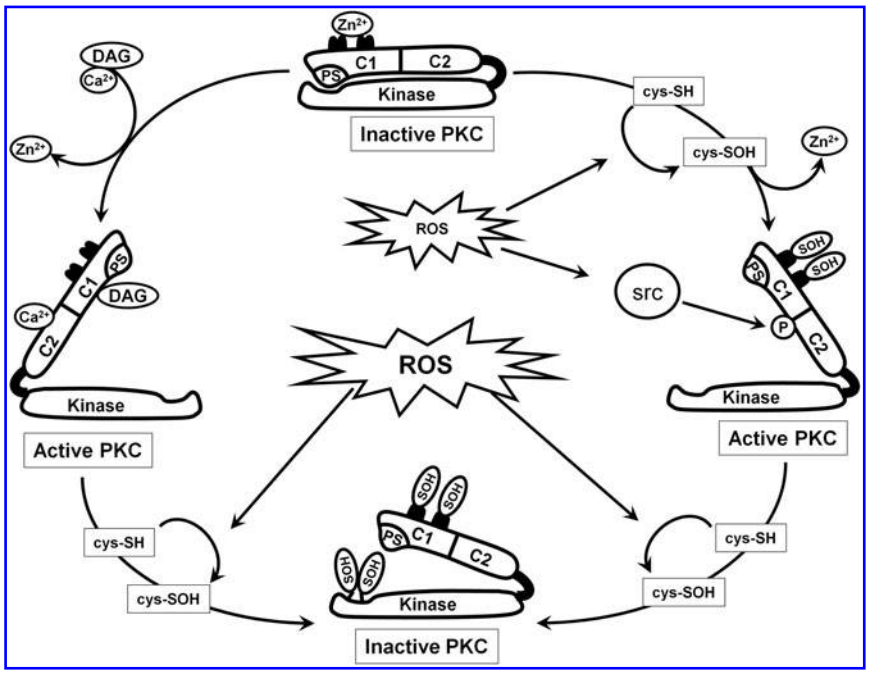

FIG. 4. Redox regulation of protein kinase C (PKC). Inactive PKC is folded so that the pseudo-substrate (PS) binds to and inhibits the kinase domain. This configuration is maintained by coordination of Zn^{2+} by four cysteine residues in the C1 domain. Canonical activation of PKC occurs through binding of Ca^{2+} and/or diacyl-glycerol (DAG) to the regulatory domain, causing release of Zn^{2+}, unfolding of PKC and kinase activation (*top left*). Alternatively, PKC is activated by low concentration ROS through oxidation of C1 cysteine residues triggering release of Zn^{2+} and activation of the kinase as before (*top right*). However, PKC may be inactivated by a stronger ROS signal through further oxidation of catalytic domain cysteine residues (*bottom*).

respond to hypoxia, both PASMC and MASMC responded to H_2O_2 and phorbol ester with enhanced PKC-ε activity and elevated $[Ca^{2+}]$i, (111, 112). The hypoxia and H_2O_2-mediated elevation of $[Ca^{2+}]$i and constriction were blocked by a PKC-ε-specific peptide inhibitor but not the conventional PKC blocker Gö6976 (111). An interpretation of these data is that the different responses to hypoxia in pulmonary and mesenteric arteries originates within the mitochondria, and not in the sensitivity of downstream pathways to ROS. However, it has been suggested that PKC may only play a role in the initial transient phase of HPV, as the wide-spectrum PKC inhibitor Ro-318220 was ineffective against sustained HPV in rat pulmonary arteries (114, 150).

PKC is likely to play a permissive role in H_2O_2-mediated endothelial cell contractility, contributing to lung edema (61, 169). Both H_2O_2-induced edema in perfused guinea-pig lung and stress fiber formation in cultured pulmonary artery endothelial cells (PAECs) were inhibited by the broad-spectrum protein kinase blocker H-7 (61), whereas the more selective PKC inhibitor calphostin C inhibited H_2O_2-mediated myosin light-chain phosphorylation in bovine PAECs but did not inhibit H_2O_2-induced stress fiber formation or recruitment of myosin to stress fibers (169), indicating a role for PKC in constriction but not actin assembly. PKC also acts downstream of NADPH oxidase-derived ROS production as part of the shear-stress-induced fluid endocytosis response in bovine aortic endothelial cells (96). Another study in bovine PAECs showed H_2O_2 causing cytosolic to membrane translocation of PKC, but also an increased production of diacylglycerol, suggesting enhanced upstream regulation of phospholipase C (140), perhaps through src/growth factor receptor activation as described above.

Direct redox activation of PKC

All PKC isozymes contain redox-sensitive cysteine residues in both regulatory and catalytic domains, which are required for autoinhibition and catalytic activity, respectively (45, 66). At low concentrations, oxidants are somehow selective for the regulatory site cysteine residues and oxidation of these residues causes intramolecular disulfide bridge formation, thus releasing the Zn^{2+} and removing the requirement for Ca^{2+}, diacylglycerol, or other lipid mediators, with disassociation of the pseudo-substrate and activation of the kinase (41, 42, 44, 45, 66, 72) (Fig. 4).

Direct redox inactivation of PKC

In contrast, PKC is inhibited by high concentrations of oxidants in both cell-free systems and living cells (44). In the catalytic domain, certain cysteine residues are required for kinase activity. These residues are selectively oxidized by antitumor oxidants such as selenite and its derivatives selenocysteine and GSSeSG, forming intramolecular disulfide bridges that inactivate the kinase through loss of catalytic activity (43). Similarly, during oxidative stress, growth-inhibiting physiological disulfides such as GSSG readily inactivate most PKC isozymes through S-thiolation, presumably due to formation of similar disulfide bridges; the exception is PKC-δ, which in contrast to other isozymes is antigrowth and proapoptotic (24, 25). Under physiological conditions, oxidized cysteine residues within the regulatory subunit of PKC are protected from reduction by cytosolic GSH by association with the plasma membrane, whereas catalytically essential cysteines in the catalytic domain are protected from oxidation by preferential reduction by thioredoxin (44) (Fig. 4).

Positive feedback between PKC activation and ROS production

As well as being activated (or inhibited) by ROS, it is well established that PKC is an important activator of most isoforms of NADPH oxidase (12). Several studies have reported that activation of PKC by various stimuli, including phorbol ester, agonists of G-protein-coupled receptors such as U46619, and pressure, all cause vasoconstriction that is mediated *via* phosphorylation of $p47^{phox}$, activation of NADPH oxidase and subsequent ROS production [*e.g.*, (49, 145)]. PKC-mediated activation of srcFK may also be involved in this process, possibly involving src-dependent promotion of rac-1 binding to the NADPH oxidase membrane subunit (49, 129). Hypoxia has also been shown to increase NADPH oxidase-derived ROS production *via* a PKC-dependent mechanism in perfused rabbit lungs (159). This is consistent with a more recent study reporting that in PASMCs a hypoxia-induced increase in mitochondrial ROS generation elicited a PKC-ε-dependent activation of NADPH oxidase, and consequently enhanced ROS production (112). This suggests that activation of both srcFK and PKC directly or indirectly by ROS can lead to amplification of the ROS signal. It also suggests that unless the sources of ROS and location of PKC/srcFK activation are strongly delimited in microdomains, there will be a positive feedback element to the response, which would have to be curtailed by inhibitory or temporal mechanisms if it were not to become pathological (150). One such mechanism might be the inhibition of PKC by high concentrations of ROS described above.

Interaction of PKC with tyrosine kinases

In the same vein as above, stimuli that elevate ROS production sometimes cause tandem activation of PKC and srcFK, and in terms of ROS signaling a reciprocal relationship between src and PKC appears to exist. On the one hand, as mentioned earlier, activation of src may be enhanced by PKC-mediated phosphorylation of src in the SH2 domain (118, 147), whereas on the other H_2O_2 promotes colocalization of several PKC isozymes with src, followed by src-dependent phosphorylation and activation of PKC (37, 44, 71, 119, 120). In addition, both srcFK and PKC may act upstream of the MAPKs ERK and p38 (see below). These complex interrelationships make interpretation of purely pharmacological studies difficult.

Mitogen-Activated Protein Kinases

MAPKs are key components of signaling pathways triggered by G-protein-coupled receptors, receptor tyrosine kinases, integrins, and cytokines. They consist of three families: ERK, p38 MAPK (p38), and jun N-terminal kinase. All MAPKs are activated through a signaling cascade starting with activation of the small G proteins ras, rac-1, or cdc42, ending with dual tyrosine/threonine phosphorylation of the MAPK. ERK is principally involved in proliferation, differentiation, and cell survival; jun N-terminal kinase in apoptosis and inflammatory responses; and p38 in cell motility and inflammatory responses (109). Of the three groups, ERK and

p38 have also been implicated in control of vascular function in response to cellular stresses such as hypoxia, oxidative stress, and inflammation, as well as to vasoconstrictor hormones (36, 64, 67, 89, 135, 149). Phosphorylation of both ERK and p38 in vascular smooth muscle and endothelium is enhanced by H_2O_2 (13, 36, 53, 89, 139, 167). This activation reportedly occurs *via* an upstream srcFK (88, 98, 99, 139, 147, 167), acting either through trans-activation of growth-factor receptors or srcFK in tandem with PKC in the case of ERK, or another un-named pathway in the case of p38 (13, 139) (Fig. 5).

MAPKs are not known to influence myosin ATPase activity directly; rather, they influence formation of the actin cytoskeleton and its interaction with myosin. ERK phosphorylates caldesmon, which in the un-phosphorylated form inhibits actin-myosin binding (32), whereas p38, through an intermediary kinase MAPKAP, phosphorylates and inhibits heat-shock protein 27 (HSP27), which in the un-phosphorylated form inhibits actin polymerization and fiber assembly (53, 135, 163). Reports vary over the relative importance of ERK and p38 to ROS-mediated smooth muscle constriction. ERK activation is implicated in ROS-dependent Ang II or stretch-mediated constriction in bovine coronary artery (36, 98), and stretch or U46619-induced NADPH oxidase-derived H_2O_2 antagonizes hypoxic relaxation of bovine coronary artery through ERK (38). ERK and p38 MAPK inhibitors block H_2O_2-mediated constriction in bovine coronary and canine cerebral arteries (83, 98) and mouse mesenteric artery (165), respectively. Ang II-mediated NADPH-dependent ROS production is required for p38 MAPK and MAPKAP activation, HSP27 phosphorylation and constriction in rat aorta, whereas MAPKAP activation and HSP27 phosphorylation and constriction were inhibited by the p38 inhibitor SB203580 (89). Conversely, in porcine pulmonary artery or mouse mesenteric arteries, H_2O_2-mediated constriction occurred independently of ERK (83, 104), whereas in bovine coronary artery, p38 inhibition was ineffectual (98). p38 may even be contributing to H_2O_2-dependent cardiac metabolic relaxation, possibly through phosphorylation of 4-aminopyridine-sensitive K^+ channels (121).

In endothelial barrier function, a greater role for p38 than for ERK is indicated. H_2O_2 enhances phosphorylation of p38 and HSP27 in human umbilical vein endothelial cells and rat PAECs (53, 54, 79, 95). In accord with this, stress fiber formation and increased endothelial cell permeability triggered by ROS-generating particulate matter or exogenous H_2O_2 is associated with enhanced p38 activity and HSP27 phosphorylation, and is blocked by the p38 blocker SB203580 or siRNA directed against either p38 or HSP27 (53, 79, 95, 149). In contrast, ERK, although activated by H_2O_2 in bovine PAECs, was not required for the associated H_2O_2-induced increases in endothelial cell permeability (54, 95).

Redox targets for MAPK activation

MAPKs are not directly redox-sensitive but instead rely on ROS-mediated activation of upstream pathways such as srcFK and PKC, as described above. However, they may also be activated through redox inhibition of upstream PTPs, or direct redox activation of ras. Redox inhibition of two PTPs, SHP-1 and HePTP, results in activation of ERK and p38, apparently through reduced de-phosphorylation of upstream MEKs, the kinases that phosphorylate and activate the two MAPKs (75). Ras, one of the monomeric G proteins that stimulate the MAPK activation cascades, contains at least one redox-sensitive cysteine residue (cys-118). When this is oxidized by s-glutathiolation in response to Ang II stimulation in vascular smooth muscle, or peroxynitrite in bovine aortic endothelial cells, ras undergoes enhanced exchange of GDP for GTP, thus enhancing activity (2, 26, 73). A similar redox mechanism exists for the related monomeric G proteins rac-1 and RhoA (see below).

Rho-Kinase

Rho-kinase is a serine/threonine kinase that is an important mediator of Ca^{2+}-sensitization and actin cytoskeleton

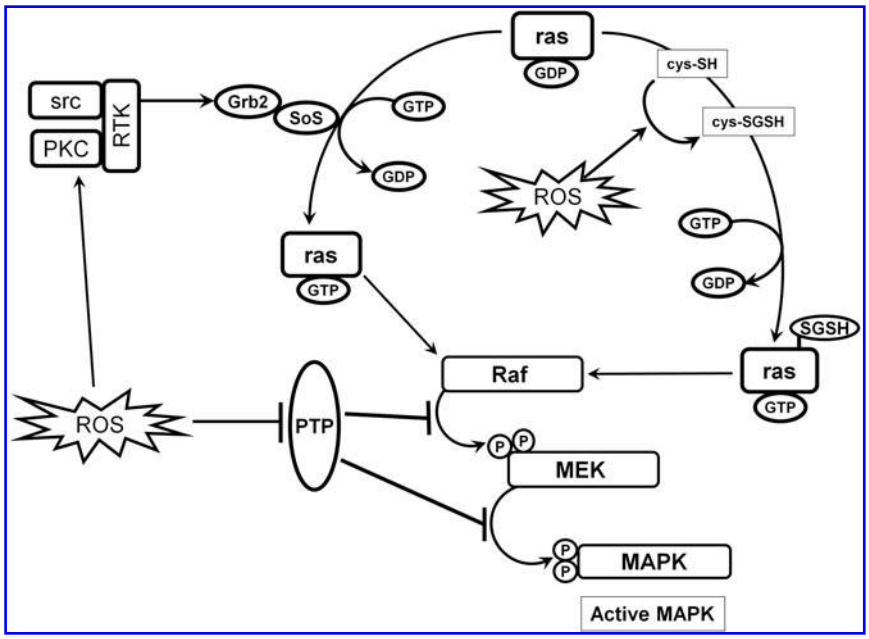

FIG. 5. Oxidative activation of mitogen-activated protein kinases (MAPK). MAPKs are activated through a cascade of phosphorylations initiated by the small G protein ras, resulting in dual tyrosine/threonine phosphorylation of the MAPK. ROS enhance this activation by inhibiting PTPs that oppose these phosphorylations (*bottom*). Alternatively, ROS may activate ras, either indirectly through activation of upstream src or PKC (*top left*) and transactivation of receptor tyrosine kinases (RTK), or by direct oxidation and glutathiolation (cys-SGSH) of cysteine residues within ras itself, promoting exchange of GDP for GTP (*top right*). Grb2, growth-factor receptor binding protein-2; SoS, son-of-sevenless (guanine exchange factor [GEF] for ras).

dynamics. It is composed of an N-terminal kinase domain, a hinge-like central coiled-coil region, and a pleckstrin-homology-like C-terminal domain. Inactive Rho-kinase is folded such that interaction between the N-terminal and C-terminal domains results in autoinhibition. Upon binding of GTP-bound RhoA to the coiled-coiled region, the kinase unfolds and becomes catalytically active (6, 87, 131). Ca^{2+} sensitization is the process by which an increase in myosin light-chain phosphorylation and hence actin-myosin cross-bridge cycling occur without a concomitant increase in intracellular Ca^{2+} (134). Rho-kinase does this through phosphorylation and inhibition of the myosin phosphatase targeting subunit (MYPT-1), resulting in enhanced myosin light-chain phosphorylation (134, 160). In addition, Rho-kinase also phosphorylates and activates Lim-kinase which in turn phosphorylates and inhibits the actin de-polymerizing chaperone cofilin, thus promoting actin polymerization and the assembly of stress fibers (84). Rho-kinase-mediated Ca^{2+} sensitization contributes to constrictor responses to various stimuli in various vascular beds, including acute responses to agonists and hypoxia in pulmonary artery (29, 56, 68, 70, 115) and during chronic hypoxia and pulmonary hypertension (14, 58, 94, 100, 132, 153).

In rat aorta and pulmonary artery, superoxide generated by xanthine/xanthine oxidase or LY83583 caused constriction and enhanced phosphorylation of MYPT-1, and both effects were blocked by the Rho-kinase inhibitor Y27632 and SOD (59, 69) (Fig. 6B). In addition, LY83583 caused Y27632-sensitive constriction of α-toxin permeabilized, Ca^{2+}-clamped pulmonary arteries and induced subcellular translocation of Rho-kinase; it did not cause elevation of Ca^{2+} in intact arteries (69). Under identical experimental conditions, LY83583 relaxed $PGF_{2\alpha}$-constricted mesenteric or femoral arteries (69). Despite this, preliminary data suggest that LY83583 causes a similar elevation of MYPT-1 phosphorylation in mesenteric as in pulmonary artery, but for some reason in mesenteric this is not coupled to constriction (own unpublished observations). In pulmonary artery from chronically hypoxic rats, increased responsiveness to both ET-1 and depolarization is attributed to enhanced Ca^{2+}-sensitization (14, 58, 132). This is associated with enhanced MYPT-1 phosphorylation and inhibited by both Y27632 and the superoxide scavenger tiron, suggesting that ROS-mediated Rho-kinase activation at least partially accounts for the effect of chronic hypoxia on pulmonary artery vasoreactivity (14, 58).

Rho-kinase is also an important mediator of ROS-induced endothelial cell permeability in experimental models of hypoxia/re-perfusion and edema. One such study on the blood–brain barrier shows that hypoxia/reperfusion-induced ROS production triggers actin polymerization, formation of contractile fibers, and actin-myosin constriction (62). In the same study, H_2O_2 also greatly increased endothelial permeability and actin polymerization, and both responses were inhibited by inhibition of Rho-kinase (62). H_2O_2 also induces pulmonary edema in perfused rabbit lung in a Y27632-inhibitable manner, suggesting that H_2O_2 activates Rho-kinase causing endothelial cell contraction and thence increased capillary permeability (21).

Redox activation of RhoA

There is no evidence that Rho-kinase is directly redox sensitive. It is therefore likely to be responding to ROS through changes to upstream signaling events. The principal activator of Rho-kinase is the small monomeric G protein RhoA, and indeed, xanthine/xanthine-oxidase-derived ROS cause constriction and cytosolic to membrane translocation of RhoA in rat aorta (59). We have observed a similar RhoA translocation response to ROS generated by LY83583 in PASMCs (Fig. 6A). RhoA is also activated by SOD-inhibitable mitochondrial superoxide in response to cold in cutaneous ar-

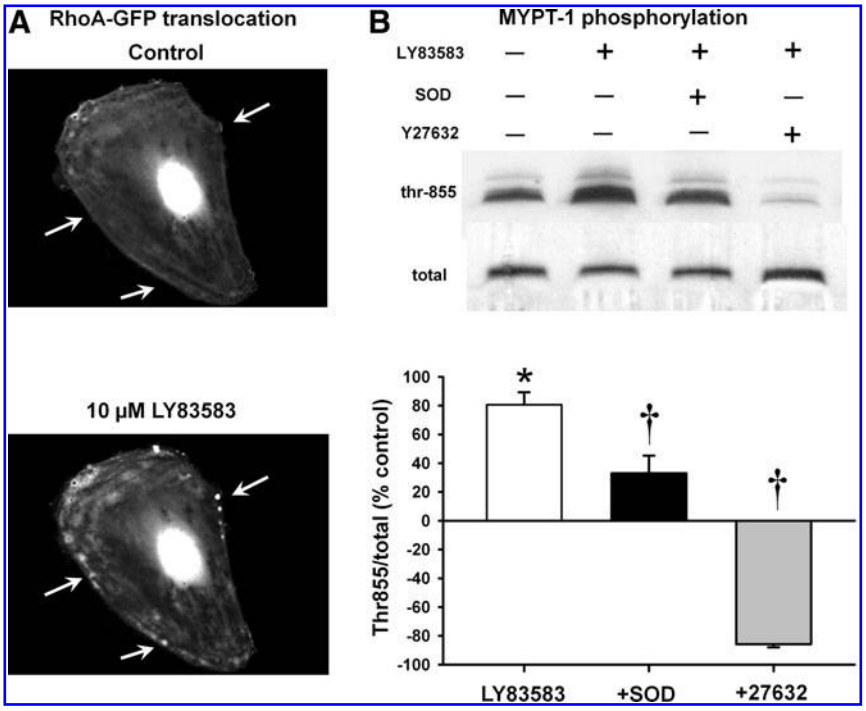

FIG. 6. Evidence for activation of Rho/Rho-kinase (ROK) pathway by superoxide in rat pulmonary artery smooth muscle. (A) Cytosolic to membrane translocation of RhoA as an indication of RhoA activation in pulmonary artery smooth muscle cells transfected with GFP-RhoA fusion protein. *Panels* show a representative cell illuminated at 490 nm before (*top*) and after (*bottom*) exposure to 10 μ*M* LY83583. Note *white spots* or *patches* appearing on cell periphery (*arrows*). **(B)** Myosin phosphatase targeting subunit (MYPT-1) phosphorylation on thr-855 in rat intrapulmonary artery is enhanced by LY83583 (*$p < 0.05$ *vs.* control) and this is inhibited by SOD or the ROK inhibitor Y27632 ($^{\dagger}p < 0.05$ *vs.* LY83583).

teries (10, 11), and by Ang II and β_2-adrenoceptor agonists *via* diphenyliodonium-sensitive NADPH oxidase-derived ROS in the renal microvasculature (55). ROS-mediated RhoA activation is also implicated in pulmonary responses to hypoxia. In chronically hypoxic rats, ET-1 and depolarization-mediated RhoA activation was enhanced compared to normoxic controls and this difference was abolished by the superoxide scavenger tiron (14, 58). Curiously, another recent study showed that after several hours of prolonged hypoxia in PASMCs and PAECs both hypoxia and H_2O_2 activated RhoA in both cell types (18). However, although an hypoxia-induced increase in ROS production was detected by a cytosol-targeted ROS indicator, the hypoxia-induced activation of RhoA was not suppressed by the antioxidant EUK-134 (18).

While it has been reported that ROS-induced activation of the Rho/Rho-kinase pathway may be mediated *via* activation of upstream tyrosine kinases (69), biochemical studies also suggest that RhoA is directly redox sensitive and is activated by ROS (3, 51). ROS and reactive nitrogen species enhance guanine nucleotide dissociation from Rho-family G proteins, including RhoA and Rac-1, and induce stress fiber formation in cultured fibroblasts (3, 51). This activation pathway required two cysteine residues in the phosphoryl binding loop of RhoA that were, nevertheless, not required for canonical guanine exchange factor-mediated RhoA activation (3). It is proposed that in living cells (where GTP is in excess) oxidation of these cysteine residues, by promoting guanine nucleotide dissociation, results in exchange of GDP for GTP, thus activating the G protein (Fig. 7).

Further Considerations

Two general observations merit further discussion: the apparent differences between vascular responses to superoxide and H_2O_2, and the possible disparity between the responses elicited by ROS generated by physiological stimuli and those elicited by ROS applied externally.

Although superoxide is the principal ROS generated by most oxidoreductase enzymes, it is generally assumed that it is rapidly converted to H_2O_2 by mitochondrial or cytosolic SOD (156). However, experimental evidence with exogenously applied ROS suggests a divergence in function between the two species. On the one hand, there is strong evidence that H_2O_2 causes constriction, activates PKC and tyrosine kinases in smooth muscle and endothelium (15, 16, 96, 108, 125, 140, 147, 165, 168, 169), and is responsible for the elevation of $[Ca^{2+}]i$ in PASMCs during hypoxia (112, 148, 156). Yet on the other hand, HPV is reportedly inhibited by SOD and enhanced by SOD antagonists (1, 82), and gene transfer of SOD, which will convert superoxide to H_2O_2, ameliorates pulmonary hypertension in rats (63), suggesting either that H_2O_2 is not procontractile, or that superoxide itself is required for constriction to develop. Rho-kinase activation is apparently superoxide specific, since both LY83583 and xanthine/xanthine-oxidase, which both generate superoxide, activate Rho-kinase in smooth muscle in a SOD-inhibitable manner (59, 69); in contrast H_2O_2 does not activate Rho-kinase, but instead activates PKC (69, 108). Such differences cannot easily be explained through subtleties of experimental technique since our studies in rat pulmonary artery show striking differences between the responses to superoxide and H_2O_2 under very similar experimental conditions (69, 108). To add to this confusion, it has been reported that RhoA activity is enhanced by both SOD-inhibitable superoxide-generating systems and exogenous H_2O_2 in smooth muscle (10, 11, 18, 59).

The concentration of H_2O_2 applied to vascular tissues certainly influences the physiological response due to compounded effects on ion channels and membrane potential as well as protein kinases (9, 39). Also, at the single molecule level, ROS may activate or inhibit different cellular targets depending on concentration, as illustrated by activation of PKC by low concentrations of ROS and inhibition by high concentrations, through selective targeting of the regulatory

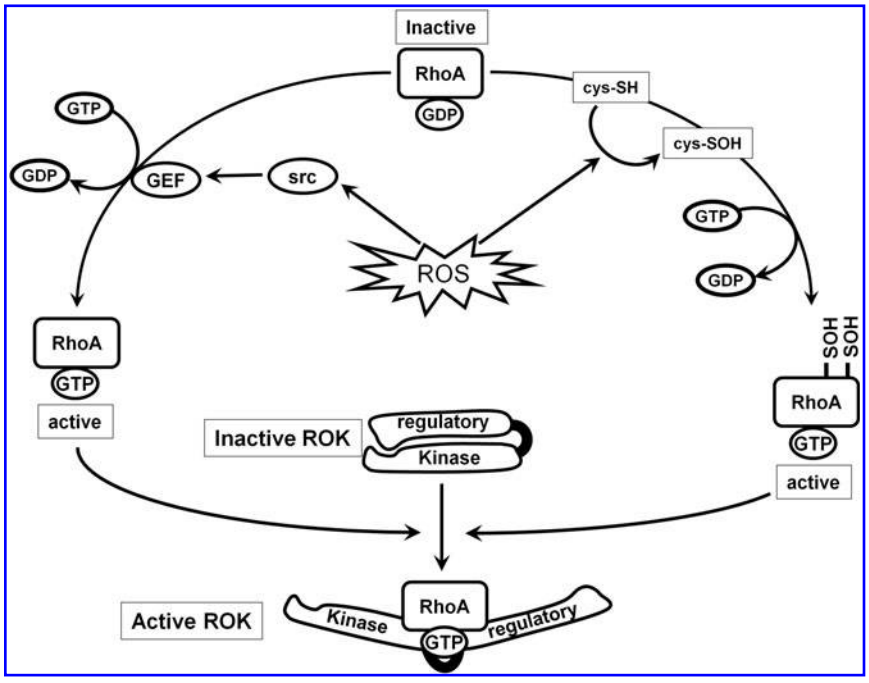

FIG. 7. Oxidative activation of ROK. Inactive ROK is folded so that the regulatory domain suppresses kinase activity. Activation occurs through binding of GTP-bound RhoA to the central hinge region, which causes ROK to unfold (*bottom*). RhoA is activated by factors that promote exchange of GDP for GTP. This is usually promoted by GEF which are also activated by ROS indirectly through src (*left*). Alternatively, RhoA is activated by cysteine residue oxidation that also promotes guanine nucleotide exchange, independently of GEF (*right*).

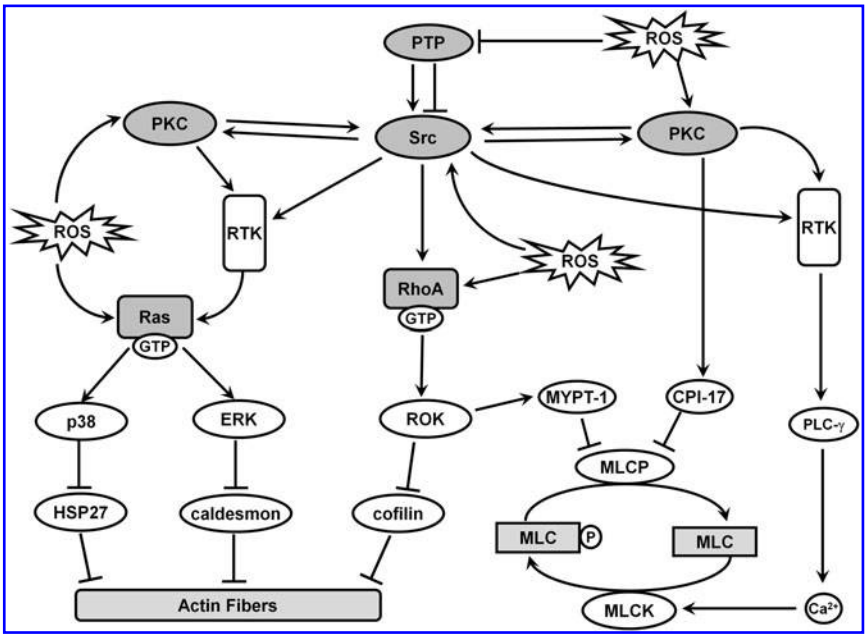

FIG. 8. Summary of redox control of protein kinases and smooth muscle constriction. PTPs are inhibited by ROS. This either activates or inhibits src. In addition, both src and PKC are directly activated by ROS and may enhance each other's activity. Both src and PKC activate ras (which is also directly redox sensitive) through trans-activation of RTK, thus leading to activation of p38 and extracellular signal-regulated kinase (ERK) MAPKs. Src and ROS also activate RhoA, which activates ROK. p38, ERK, and ROK all promote formation of actin fibers, and/or their association with myosin, through inhibition of heat-shock protein-27 (HSP-27), caldesmon, and cofilin, respectively. Constriction is promoted through RTK-mediated activation of phospholipase C (PLC-γ) and subsequent IP_3-dependent release of Ca^{2+}, which activates myosin light-chain kinase (MLCK) and by ROK-dependent phosphorylation of MYPT-1 or PKC-dependent phosphorylation of CPI-17, two myosin binding proteins that inhibit myosin light-chain phosphatase (MLCP).

and catalytic domains, respectively (42–44). The lower the pKa, the more susceptible a cysteine is likely to be to a weaker oxidizing environment, whereas cysteines with higher pKa may only be affected by excessive oxidative stress (105, 143). This point is raised by Janssen-Heininger *et al.*, who suggest that care should be taken when interpreting data taken from experiments with exogenously applied oxidants such as H_2O_2, particularly when applied at supra-physiological concentrations (>m*M*). Mild oxidants generated at or near the target proteins are more likely to form a part of tightly controlled highly localized signaling events, whereas strong oxidants may oxidize amino acid residues of multiple proteins indiscriminately (57).

Similarly, externally applied H_2O_2 even at physiological concentrations is likely to activate multiple pathways simultaneously in an unregulated fashion, thus generating cellular responses that do not necessarily match those observed in response to physiological stimuli that may or may not include redox signaling. Because ROS are normally short-lived, it is suggested that for signaling purposes the site of ROS production is localized to the same subcellular compartment or protein complex as the target protein (146). This also provides the potential for selective activation of specific signaling systems in distinct subcellular regions (17, 52, 162).

Conclusion

As summarized in Figure 8, there is considerable accumulated evidence that all of the major classes of protein kinases that play a role in smooth muscle constriction and/or endothelial permeability are redox sensitive, either through direct oxidative modification of cysteine residues or indirectly through other upstream kinases, small G proteins, or tyrosine phosphatases. Further, it is clear that these kinases play a major role in mediating altered vascular function in response to physiological and pathological stimuli that elevate ROS production. Notwithstanding the complexity of interactions between the various protein kinases, and between them and sources of ROS such as NADPH oxidase and mitochondria, it is, nevertheless, clear that protein kinases play a central role in ROS signaling in the vasculature, and that the latter is a key physiological modulator of vascular function. Future work is likely to focus on unraveling the potential importance of ROS-signaling microdomains containing specific target mechanisms. It is only recently that the tools necessary for this have started to become available [*e.g.*, (155)].

Acknowledgments

Thanks to Vladimir Snetkov and Yasin Shaifta for assistance with RhoA-GFP translocation studies. We also thank the British Heart Foundation and the Wellcome Trust for financial support.

References

1. Abdalla S and Will JA. Potentiation of the hypoxic contraction of guinea-pig isolated pulmonary arteries by two inhibitors of superoxide dismutase. *Gen Pharmacol* 26: 785–792, 1995.
2. Adachi T, Pimentel DR, Heibeck T, Hou X, Lee YJ, Jiang B, *et al.* S-glutathiolation of Ras mediates redox-sensitive signaling by angiotensin II in vascular smooth muscle cells. *J Biol Chem* 279: 29857–29862, 2004.
3. Aghajanian A, Wittchen ES, Campbell SL, and Burridge K. Direct activation of RhoA by reactive oxygen species requires a redox-sensitive motif. *PLoS ONE* 4: e8045, 2009.
4. Ahsan MK, Lekli I, Ray D, Yodoi J, and Das DK. Redox regulation of cell survival by the thioredoxin superfamily: an implication of redox gene therapy in the heart. *Antioxid Redox Signal* 11: 2741–2758, 2009.

5. Akhand AA, Pu M, Senga T, Kato M, Suzuki H, Miyata T, *et al.* Nitric oxide controls src kinase activity through a sulfhydryl group modification-mediated Tyr-527-independent and Tyr-416-linked mechanism. *J Biol Chem* 274: 25821–25826, 1999.
6. Amano M, Fukata Y, and Kaibuchi K. Regulation and functions of Rho-associated kinase. *Exp Cell Res* 261: 44–51, 2000.
7. Archer SL, Michelakis ED, Thebaud B, Bonnet S, Moudgil R, Wu XC, *et al.* A central role for oxygen-sensitive K+ channels and mitochondria in the specialized oxygen-sensing system. *Novartis Found Symp* 272: 157–171, 2006.
8. Ardanaz N, Beierwaltes WH, and Pagano PJ. Comparison of H_2O_2-induced vasoconstriction in the abdominal aorta and mesenteric artery of the mouse. *Vascul Pharmacol* 47: 288–294, 2007.
9. Bae ON, Lim KM, Han JY, Jung BI, Lee JY, Noh JY, *et al.* U-shaped dose response in vasomotor tone: a mixed result of heterogenic response of multiple cells to xenobiotics. *Toxicol Sci* 103: 181–190, 2008.
10. Bailey SR, Eid AH, Mitra S, Flavahan S, and Flavahan NA. Rho kinase mediates cold-induced constriction of cutaneous arteries: role of alpha2C-adrenoceptor translocation. *Circ Res* 94: 1367–1374, 2004.
11. Bailey SR, Mitra S, Flavahan S, and Flavahan NA. Reactive oxygen species from smooth muscle mitochondria initiate cold-induced constriction of cutaneous arteries. *Am J Physiol Heart Circ Physiol* 289: H243–H250, 2005.
12. Bedard K and Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 87: 245–313, 2007.
13. Blanc A, Pandey NR, and Srivastava AK. Distinct roles of Ca2+, calmodulin, and protein kinase C in H_2O_2-induced activation of ERK1/2, p38 MAPK, and protein kinase B signaling in vascular smooth muscle cells. *Antioxid Redox Signal* 6: 353–366, 2004.
14. Broughton BR, Jernigan NL, Norton CE, Walker BR, and Resta TC. Chronic hypoxia augments depolarization-induced Ca2+-sensitization in pulmonary vascular smooth muscle through superoxide-dependent stimulation of RhoA. *Am J Physiol Lung Cell Mol Physiol* 298: L232–L242, 2010.
15. Chakraborti S and Chakraborti T. Down-regulation of protein kinase C attenuates the oxidant hydrogen peroxide-mediated activation of phospholipase A2 in pulmonary vascular smooth muscle cells. *Cell Signal* 7: 75–83, 1995.
16. Chakraborti S and Michael JR. Role of protein kinase C in oxidant-mediated activation of phospholipase A2 in rabbit pulmonary arterial smooth muscle cells. *Mol Cell Biochem* 122: 9–15, 1993.
17. Chen K, Kirber MT, Xiao H, Yang Y, and Keaney JF Jr. Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J Cell Biol* 181: 1129–1139, 2008.
18. Chi AY, Waypa GB, Mungai PT, and Schumacker PT. Prolonged hypoxia increases ROS signaling and RhoA activation in pulmonary artery smooth muscle and endothelial cells. *Antioxid Redox Signal* 12: 603–610, 2010.
19. Chiang GG and Sefton BM. Phosphorylation of a Src kinase at the autophosphorylation site in the absence of Src kinase activity. *J Biol Chem* 275: 6055–6058, 2000.
20. Chiarugi P, Pani G, Giannoni E, Taddei L, Colavitti R, Raugei G, *et al.* Reactive oxygen species as essential mediators of cell adhesion: the oxidative inhibition of a FAK tyrosine phosphatase is required for cell adhesion. *J Cell Biol* 161: 933–944, 2003.
21. Chiba Y, Ishii Y, Kitamura S, and Sugiyama Y. Activation of rho is involved in the mechanism of hydrogen-peroxide-induced lung edema in isolated perfused rabbit lung. *Microvasc Res* 62: 164–171, 2001.
22. Chopra LC, Hucks D, Twort CH, and Ward JP. Effects of protein tyrosine kinase inhibitors on contractility of isolated bronchioles of the rat. *Am J Respir Cell Mol Biol* 16: 372–378, 1997.
23. Chowdhury AK, Watkins T, Parinandi NL, Saatian B, Kleinberg ME, Usatyuk PV, *et al.* Src-mediated tyrosine phosphorylation of p47phox in hyperoxia-induced activation of NADPH oxidase and generation of reactive oxygen species in lung endothelial cells. *J Biol Chem* 280: 20700–20711, 2005.
24. Chu F, Chen LH, and O'Brian CA. Cellular protein kinase C isozyme regulation by exogenously delivered physiological disulfides—implications of oxidative protein kinase C regulation to cancer prevention. *Carcinogenesis* 25: 585–596, 2004.
25. Chu F, Ward NE, and O'Brian CA. Potent inactivation of representative members of each PKC isozyme subfamily and PKD via S-thiolation by the tumor-promotion/progression antagonist glutathione but not by its precursor cysteine. *Carcinogenesis* 22: 1221–1229, 2001.
26. Clavreul N, Adachi T, Pimental DR, Ido Y, Schoneich C, and Cohen RA. S-glutathiolation by peroxynitrite of p21ras at cysteine-118 mediates its direct activation and downstream signaling in endothelial cells. *FASEB J* 20: 518–520, 2006.
27. Cooper JA, Gould KL, Cartwright CA, and Hunter T. Tyr527 is phosphorylated in pp60c-src: implications for regulation. *Science* 231: 1431–1434, 1986.
28. Cruzado MC, Risler NR, Miatello RM, Yao G, Schiffrin EL, and Touyz RM. Vascular smooth muscle cell NAD(P)H oxidase activity during the development of hypertension: effect of angiotensin II and role of insulinlike growth factor-1 receptor transactivation. *Am J Hypertens* 18: 81–87, 2005.
29. Damron DS, Kanaya N, Homma Y, Kim SO, and Murray PA. Role of PKC, tyrosine kinases, and Rho kinase in alpha-adrenoreceptor-mediated PASM contraction. *Am J Physiol Lung Cell Mol Physiol* 283: L1051–L1064, 2002.
30. De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, and Griendling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. *Circ Res* 82: 1094–1101, 1998.
31. Denu JM and Tanner KG. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* 37: 5633–5642, 1998.
32. Dessy C, Kim I, Sougnez CL, Laporte R, and Morgan KG. A role for MAP kinase in differentiated smooth muscle contraction evoked by alpha-adrenoceptor stimulation. *Am J Physiol* 275: C1081–C1086, 1998.
33. Di SJ, Semenchuk LA, and Lauer J. Vanadate-induced contraction of smooth muscle and enhanced protein tyrosine phosphorylation. *Arch Biochem Biophys* 304: 386–391, 1993.
34. Erdos B, Snipes JA, Miller AW, and Busija DW. Cerebrovascular dysfunction in Zucker obese rats is mediated by oxidative stress and protein kinase C. *Diabetes* 53: 1352–1359, 2004.
35. Forstermann U. Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. *Nat Clin Pract Cardiovasc Med* 5: 338–349, 2008.

36. Frank GD, Eguchi S, Yamakawa T, Tanaka S, Inagami T, and Motley ED. Involvement of reactive oxygen species in the activation of tyrosine kinase and extracellular signal-regulated kinase by angiotensin II. *Endocrinology* 141: 3120–3126, 2000.
37. Fukunaga M, Oka M, Ichihashi M, Yamamoto T, Matsuzaki H, and Kikkawa U. UV-induced tyrosine phosphorylation of PKC delta and promotion of apoptosis in the HaCaT cell line. *Biochem Biophys Res Commun* 289: 573–579, 2001.
38. Gao Q, Zhao X, Ahmad M, and Wolin MS. Mitochondrial-derived hydrogen peroxide inhibits relaxation of bovine coronary arterial smooth muscle to hypoxia through stimulation of ERK MAP kinase. *Am J Physiol Heart Circ Physiol* 297: H2262–H2269, 2009.
39. Gao YJ, Hirota S, Zhang DW, Janssen LJ, and Lee RM. Mechanisms of hydrogen-peroxide-induced biphasic response in rat mesenteric artery. *Br J Pharmacol* 138: 1085–1092, 2003.
40. Giannoni E, Buricchi F, Raugei G, Ramponi G, and Chiarugi P. Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. *Mol Cell Biol* 25: 6391–6403, 2005.
41. Gopalakrishna R and Anderson WB. Ca2+- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain. *Proc Natl Acad Sci U S A* 86: 6758–6762, 1989.
42. Gopalakrishna R, Chen ZH, and Gundimeda U. Modifications of cysteine-rich regions in protein kinase C induced by oxidant tumor promoters and enzyme-specific inhibitors. *Methods Enzymol* 252: 132–146, 1995.
43. Gopalakrishna R, Gundimeda U, and Chen ZH. Cancer-preventive selenocompounds induce a specific redox modification of cysteine-rich regions in Ca(2+)-dependent isoenzymes of protein kinase C. *Arch Biochem Biophys* 348: 25–36, 1997.
44. Gopalakrishna R, Gundimeda U, Schiffman JE, and McNeill TH. A direct redox regulation of protein kinase C isoenzymes mediates oxidant-induced neuritogenesis in PC12 cells. *J Biol Chem* 283: 14430–14444, 2008.
45. Gopalakrishna R and Jaken S. Protein kinase C signaling and oxidative stress. *Free Radic Biol Med* 28: 1349–1361, 2000.
46. Griendling KK, Minieri CA, Ollerenshaw JD, and Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 74: 1141–1148, 1994.
47. Griendling KK, Sorescu D, Lassegue B, and Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler Thromb Vasc Biol* 20: 2175–2183, 2000.
48. Griendling KK and Ushio-Fukai M. Reactive oxygen species as mediators of angiotensin II signaling. *Regul Pept* 91: 21–27, 2000.
49. Gupte SA, Kaminski PM, George S, Kouznestova L, Olson SC, Mathew R, *et al.* Peroxide generation by p47phox-Src activation of Nox2 has a key role in protein kinase C-induced arterial smooth muscle contraction. *Am J Physiol Heart Circ Physiol* 296: H1048–H1057, 2009.
50. Hasegawa T, Bando A, Tsuchiya K, Abe S, Okamoto M, Kirima K, *et al.* Enzymatic and nonenzymatic formation of reactive oxygen species from 6-anilino-5,8-quinolinequinone. *Biochim Biophys Acta* 1670: 19–27, 2004.
51. Heo J and Campbell SL. Mechanism of redox-mediated guanine nucleotide exchange on redox-active Rho GTPases. *J Biol Chem* 280: 31003–31010, 2005.
52. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, and Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 24: 677–683, 2004.
53. Hirano S, Rees RS, Yancy SL, Welsh MJ, Remick DG, Yamada T, *et al.* Endothelial barrier dysfunction caused by LPS correlates with phosphorylation of HSP27 *in vivo*. *Cell Biol Toxicol* 20: 1–14, 2004.
54. Huot J, Houle F, Marceau F, and Landry J. Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circ Res* 80: 383–392, 1997.
55. Jackson EK, Gillespie DG, Zhu C, Ren J, Zacharia LC, and Mi Z. Alpha2-adrenoceptors enhance angiotensin II-induced renal vasoconstriction: role for NADPH oxidase and RhoA. *Hypertension* 51: 719–726, 2008.
56. Janssen LJ, Lu-Chao H, and Netherton S. Excitation-contraction coupling in pulmonary vascular smooth muscle involves tyrosine kinase and Rho kinase. *Am J Physiol Lung Cell Mol Physiol* 280: L666–L674, 2001.
57. Janssen-Heininger YM, Mossman BT, Heintz NH, Forman HJ, Kalyanaraman B, Finkel T, *et al.* Redox-based regulation of signal transduction: principles, pitfalls, and promises. *Free Radic Biol Med* 45: 1–17, 2008.
58. Jernigan NL, Walker BR, and Resta TC. Reactive oxygen species mediate RhoA/Rho kinase-induced Ca2+ sensitization in pulmonary vascular smooth muscle following chronic hypoxia. *Am J Physiol Lung Cell Mol Physiol* 295: L515–L529, 2008.
59. Jin L, Ying Z, and Webb RC. Activation of Rho/Rho kinase signaling pathway by reactive oxygen species in rat aorta. *Am J Physiol Heart Circ Physiol* 287: H1495–H1500, 2004.
60. Jin N and Rhoades RA. Activation of tyrosine kinases in H_2O_2-induced contraction in pulmonary artery. *Am J Physiol* 272: H2686–H2692, 1997.
61. Johnson A, Phillips P, Hocking D, Tsan MF, and Ferro T. Protein kinase inhibitor prevents pulmonary edema in response to H_2O_2. *Am J Physiol* 256: H1012–H1022, 1989.
62. Kahles T, Luedike P, Endres M, Galla HJ, Steinmetz H, Busse R, *et al.* NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke. *Stroke* 38: 3000–3006, 2007.
63. Kamezaki F, Tasaki H, Yamashita K, Tsutsui M, Koide S, Nakata S, *et al.* Gene transfer of extracellular superoxide dismutase ameliorates pulmonary hypertension in rats. *Am J Respir Crit Care Med* 177: 219–226, 2008.
64. Karamsetty MR, Klinger JR, and Hill NS. Evidence for the role of p38 MAP kinase in hypoxia-induced pulmonary vasoconstriction. *Am J Physiol Lung Cell Mol Physiol* 283: L859–L866, 2002.
65. Kemble DJ and Sun G. Direct and specific inactivation of protein tyrosine kinases in the Src and FGFR families by reversible cysteine oxidation. *Proc Natl Acad Sci U S A* 106: 5070–5075, 2009.
66. Knapp LT and Klann E. Superoxide-induced stimulation of protein kinase C via thiol modification and modulation of zinc content. *J Biol Chem* 275: 24136–24145, 2000.
67. Knock GA, De Silva AS, Snetkov VA, Siow R, Thomas GD, Shiraishi M, *et al.* Modulation of PGF2- and hypoxia-induced contraction of rat intrapulmonary artery by p38 MAPK inhibition: a nitric oxide-dependent mechanism. *Am J Physiol Lung Cell Mol Physiol* 289: L1039–L1048, 2005.
68. Knock GA, Shaifta Y, Snetkov VA, Vowles B, Drndarski S, Ward JP, *et al.* Interaction between src family kinases and

rho-kinase in agonist-induced Ca2+-sensitization of rat pulmonary artery. *Cardiovasc Res* 77: 570–579, 2008.
69. Knock GA, Snetkov VA, Shaifta Y, Connolly M, Drndarski S, Noah A, *et al.* Superoxide constricts rat pulmonary arteries via Rho-kinase-mediated Ca(2+) sensitization. *Free Radic Biol Med* 46: 633–642, 2009.
70. Knock GA, Snetkov VA, Shaifta Y, Drndarski S, Ward JP, and Aaronson PI. Role of src-family kinases in hypoxic vasoconstriction of rat pulmonary artery. *Cardiovasc Res* 80: 453–462, 2008.
71. Konishi H, Tanaka M, Takemura Y, Matsuzaki H, Ono Y, Kikkawa U, *et al.* Activation of protein kinase C by tyrosine phosphorylation in response to H_2O_2. *Proc Natl Acad Sci U S A* 94: 11233–11237, 1997.
72. Korichneva I, Hoyos B, Chua R, Levi E, and Hammerling U. Zinc release from protein kinase C as the common event during activation by lipid second messenger or reactive oxygen. *J Biol Chem* 277: 44327–44331, 2002.
73. Lander HM, Hajjar DP, Hempstead BL, Mirza UA, Chait BT, Campbell S, *et al.* A molecular redox switch on p21(ras). Structural basis for the nitric oxide-p21(ras) interaction. *J Biol Chem* 272: 4323–4326, 1997.
74. Laniyonu AA, Saifeddine M, Yang SG, and Hollenberg MD. Tyrosine kinase inhibitors and the contractile action of G-protein-linked vascular agonists. *Can J Physiol Pharmacol* 72: 1075–1085, 1994.
75. Lee K and Esselman WJ. Inhibition of PTPs by H_2O_2 regulates the activation of distinct MAPK pathways. *Free Radic Biol Med* 33: 1121–1132, 2002.
76. Lee SR, Kwon KS, Kim SR, and Rhee SG. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* 273: 15366–15372, 1998.
77. Li Q, Zhang Y, Marden JJ, Banfi B, and Engelhardt JF. Endosomal NADPH oxidase regulates c-Src activation following hypoxia/reoxygenation injury. *Biochem J* 411: 531–541, 2008.
78. Li W, Mischak H, Yu JC, Wang LM, Mushinski JF, Heidaran MA, *et al.* Tyrosine phosphorylation of protein kinase C-delta in response to its activation. *J Biol Chem* 269: 2349–2352, 1994.
79. Li Z, Hyseni X, Carter JD, Soukup JM, Dailey LA, and Huang YC. Pollutant particles enhanced H_2O_2 production from NAD(P)H oxidase and mitochondria in human pulmonary artery endothelial cells. *Am J Physiol Cell Physiol* 291: C357–C365, 2006.
80. Liaudet L, Vassalli G, and Pacher P. Role of peroxynitrite in the redox regulation of cell signal transduction pathways. *Front Biosci* 14: 4809–4814, 2009.
81. Littler CM, Morris KG Jr., Fagan KA, McMurtry IF, Messing RO, and Dempsey EC. Protein kinase C-epsilon-null mice have decreased hypoxic pulmonary vasoconstriction. *Am J Physiol Heart Circ Physiol* 284: H1321–H1331, 2003.
82. Liu JQ, Sham JS, Shimoda LA, Kuppusamy P, and Sylvester JT. Hypoxic constriction and reactive oxygen species in porcine distal pulmonary arteries. *Am J Physiol Lung Cell Mol Physiol* 285: L322–L333, 2003.
83. Lucchesi PA, Belmadani S, and Matrougui K. Hydrogen peroxide acts as both vasodilator and vasoconstrictor in the control of perfused mouse mesenteric resistance arteries. *J Hypertens* 23: 571–579, 2005.
84. Maekawa M, Ishizaki T, Boku S, Watanabe N, Fujita A, Iwamatsu A, *et al.* Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 285: 895–898, 1999.
85. Mallozzi C, Di Stasi AM, and Minetti M. Nitrotyrosine mimics phosphotyrosine binding to the SH2 domain of the src family tyrosine kinase lyn. *FEBS Lett* 503: 189–195, 2001.
86. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, *et al.* Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* 106: 1521–1530, 2000.
87. Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, *et al.* Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J* 15: 2208–2216, 1996.
88. Mehdi MZ, Pandey NR, Pandey SK, and Srivastava AK. H_2O_2-induced phosphorylation of ERK1/2 and PKB requires tyrosine kinase activity of insulin receptor and c-Src. *Antioxid Redox Signal* 7: 1014–1020, 2005.
89. Meloche S, Landry J, Huot J, Houle F, Marceau F, and Giasson E. p38 MAP kinase pathway regulates angiotensin II-induced contraction of rat vascular smooth muscle. *Am J Physiol Heart Circ Physiol* 279: H741–H751, 2000.
90. Mills JE, Whitford PC, Shaffer J, Onuchic JN, Adams JA, and Jennings PA. A novel disulfide bond in the SH2 Domain of the C-terminal Src kinase controls catalytic activity. *J Mol Biol* 365: 1460–1468, 2007.
91. Minetti M, Mallozzi C, and Di Stasi AM. Peroxynitrite activates kinases of the src family and upregulates tyrosine phosphorylation signaling. *Free Radic Biol Med* 33: 744–754, 2002.
92. Mohazzab KM, Kaminski PM, and Wolin MS. NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am J Physiol* 266: H2568–H2572, 1994.
93. Moudgil R, Michelakis ED, and Archer SL. Hypoxic pulmonary vasoconstriction. *J Appl Physiol* 98: 390–403, 2005.
94. Nagaoka T, Morio Y, Casanova N, Bauer N, Gebb S, McMurtry I, *et al.* Rho/Rho kinase signaling mediates increased basal pulmonary vascular tone in chronically hypoxic rats. *Am J Physiol Lung Cell Mol Physiol* 287: L665–L672, 2004.
95. Niwa K, Inanami O, Ohta T, Ito S, Karino T, and Kuwabara M. p38 MAPK and Ca2+ contribute to hydrogen peroxide-induced increase of permeability in vascular endothelial cells but ERK does not. *Free Radic Res* 35: 519–527, 2001.
96. Niwa K, Sakai J, Karino T, Aonuma H, Watanabe T, Ohyama T, *et al.* Reactive oxygen species mediate shear stress-induced fluid-phase endocytosis in vascular endothelial cells. *Free Radic Res* 40: 167–174, 2006.
97. Oeckler RA, Arcuino E, Ahmad M, Olson SC, and Wolin MS. Cytosolic NADH redox and thiol oxidation regulate pulmonary arterial force through ERK MAP kinase. *Am J Physiol Lung Cell Mol Physiol* 288: L1017–L1025, 2005.
98. Oeckler RA, Kaminski PM, and Wolin MS. Stretch enhances contraction of bovine coronary arteries via an NAD(P)H oxidase-mediated activation of the extracellular signal-regulated kinase mitogen-activated protein kinase cascade. *Circ Res* 92: 23–31, 2003.
99. Ohanian J, Cunliffe P, Ceppi E, Alder A, Heerkens E, and Ohanian V. Activation of p38 mitogen-activated protein kinases by endothelin and noradrenaline in small arteries, regulation by calcium influx and tyrosine kinases, and their role in contraction. *Arterioscler Thromb Vasc Biol* 21: 1921–1927, 2001.
100. Oka M, Homma N, and McMurtry IF. Rho kinase-mediated vasoconstriction in rat models of pulmonary hypertension. *Methods Enzymol* 439: 191–204, 2008.

101. Okatani Y, Watanabe K, Wakatsuki A, and Sagara Y. Vasospastic action of hydrogen peroxide in human umbilical artery: relation to protein kinase C and calcium influx. *Acta Obstet Gynecol Scand* 76: 897–902, 1997.
102. Patterson C, Ruef J, Madamanchi NR, Barry-Lane P, Hu Z, Horaist C, *et al.* Stimulation of a vascular smooth muscle cell NAD(P)H oxidase by thrombin. Evidence that p47(phox) may participate in forming this oxidase *in vitro* and *in vivo*. *J Biol Chem* 274: 19814–19822, 1999.
103. Pelaez NJ, Braun TR, Paul RJ, Meiss RA, and Packer CS. H_2O_2 mediates Ca2+- and MLC20 phosphorylation-independent contraction in intact and permeabilized vascular muscle. *Am J Physiol Heart Circ Physiol* 279: H1185–H1193, 2000.
104. Pelaez NJ, Osterhaus SL, Mak AS, Zhao Y, Davis HW, and Packer CS. MAPK and PKC activity are not required for H_2O_2-induced arterial muscle contraction. *Am J Physiol Heart Circ Physiol* 279: H1194–H1200, 2000.
105. Peters GH, Frimurer TM, and Olsen OH. Electrostatic evaluation of the signature motif (H/V)CX5R(S/T) in protein-tyrosine phosphatases. *Biochemistry* 37: 5383–5393, 1998.
106. Petrone A and Sap J. Emerging issues in receptor protein tyrosine phosphatase function: lifting fog or simply shifting? *J Cell Sci* 113: 2345–2354, 2000.
107. Peus D, Vasa RA, Meves A, Pott M, Beyerle A, Squillace K, *et al.* H_2O_2 is an important mediator of UVB-induced EGF-receptor phosphorylation in cultured keratinocytes. *J Invest Dermatol* 110: 966–971, 1998.
108. Pourmahram GE, Snetkov VA, Shaifta Y, Drndarski S, Knock GA, Aaronson PI, *et al.* Constriction of pulmonary artery by peroxide: role of Ca2+ release and PKC. *Free Radic Biol Med* 45: 1468–1476, 2008.
109. Qi M and Elion EA. MAP kinase pathways. *J Cell Sci* 118: 3569–3572, 2005.
110. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, *et al.* Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 97: 1916–1923, 1996.
111. Rathore R, Zheng YM, Li XQ, Wang QS, Liu QH, Ginnan R, *et al.* Mitochondrial ROS-PKC-signaling axis is uniquely involved in hypoxic increase in [Ca2+]i in pulmonary artery smooth muscle cells. *Biochem Biophys Res Commun* 351: 784–790, 2006.
112. Rathore R, Zheng YM, Niu CF, Liu QH, Korde A, Ho YS, *et al.* Hypoxia activates NADPH oxidase to increase [ROS]i and [Ca2+]i through the mitochondrial ROS-PKC- signaling axis in pulmonary artery smooth muscle cells. *Free Radic Biol Med* 45: 1223–1231, 2008.
113. Rhoades RA, Packer CS, Roepke DA, Jin N, and Meiss RA. Reactive oxygen species alter contractile properties of pulmonary arterial smooth muscle. *Can J Physiol Pharmacol* 68: 1581–1589, 1990.
114. Robertson TP, Aaronson PI, and Ward JP. Hypoxic vasoconstriction and intracellular Ca2+ in pulmonary arteries: evidence for PKC-independent Ca2+ sensitization. *Am J Physiol* 268: H301–H307, 1995.
115. Robertson TP, Dipp M, Ward JP, Aaronson PI, and Evans AM. Inhibition of sustained hypoxic vasoconstriction by Y-27632 in isolated intrapulmonary arteries and perfused lung of the rat. *Br J Pharmacol* 131: 5–9, 2000.
116. Rogers PA, Chilian WM, Bratz IN, Bryan RM Jr., and Dick GM. H_2O_2 activates redox- and 4-aminopyridine-sensitive Kv channels in coronary vascular smooth muscle. *Am J Physiol Heart Circ Physiol* 292: H1404–H1411, 2007.
117. Rosado JA, Redondo PC, Salido GM, Gomez-Arteta E, Sage SO, and Pariente JA. Hydrogen peroxide generation induces pp60src activation in human platelets: evidence for the involvement of this pathway in store-mediated calcium entry. *J Biol Chem* 279: 1665–1675, 2004.
118. Roskoski R Jr. Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* 331: 1–14, 2005.
119. Rybin VO, Guo J, Sabri A, Elouardighi H, Schaefer E, and Steinberg SF. Stimulus-specific differences in protein kinase C delta localization and activation mechanisms in cardiomyocytes. *J Biol Chem* 279: 19350–19361, 2004.
120. Saito S, Frank GD, Mifune M, Ohba M, Utsunomiya H, Motley ED, *et al.* Ligand-independent trans-activation of the platelet-derived growth factor receptor by reactive oxygen species requires protein kinase C-delta and c-Src. *J Biol Chem* 277: 44695–44700, 2002.
121. Saitoh S, Kiyooka T, Rocic P, Rogers PA, Zhang C, Swafford A, *et al.* Redox-dependent coronary metabolic dilation. *Am J Physiol Heart Circ Physiol* 293: H3720–H3725, 2007.
122. Saitoh S, Zhang C, Tune JD, Potter B, Kiyooka T, Rogers PA, *et al.* Hydrogen peroxide: a feed-forward dilator that couples myocardial metabolism to coronary blood flow. *Arterioscler Thromb Vasc Biol* 26: 2614–2621, 2006.
123. Salsman SJ, Hensley K, and Floyd RA. Sensitivity of protein tyrosine phosphatase activity to the redox environment, cytochrome C, and microperoxidase. *Antioxid Redox Signal* 7: 1078–1088, 2005.
124. Sarsour EH, Kumar MG, Chaudhuri L, Kalen AL, and Goswami PC. Redox control of the cell cycle in health and disease. *Antioxid Redox Signal* 11: 2985–3011, 2009.
125. Sato H, Sato M, Kanai H, Uchiyama T, Iso T, Ohyama Y, *et al.* Mitochondrial reactive oxygen species and c-Src play a critical role in hypoxic response in vascular smooth muscle cells. *Cardiovasc Res* 67: 714–722, 2005.
126. Satoh K, Nigro P, and Berk BC. Oxidative stress and vascular smooth muscle cell growth: a mechanistic linkage by cyclophilin A. *Antioxid Redox Signal* 12: 675–682, 2010.
127. Schulz E, Jansen T, Wenzel P, Daiber A, and Munzel T. Nitric oxide, tetrahydrobiopterin, oxidative stress, and endothelial dysfunction in hypertension. *Antioxid Redox Signal* 10: 1115–1126, 2008.
128. Serfilippi G, Ferro TJ, and Johnson A. Activation of protein kinase C mediates altered pulmonary vasoreactivity induced by tumor necrosis factor-alpha. *Am J Physiol* 267: L282–L290, 1994.
129. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, and Griendling KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 91: 406–413, 2002.
130. Sheehan DW, Giese EC, Gugino SF, and Russell JA. Characterization and mechanisms of H_2O_2-induced contractions of pulmonary arteries. *Am J Physiol* 264: H1542–H1547, 1993.
131. Shimizu T, Ihara K, Maesaki R, Amano M, Kaibuchi K, and Hakoshima T. Parallel coiled-coil association of the RhoA-binding domain in Rho-kinase. *J Biol Chem* 278: 46046–46051, 2003.
132. Shimoda LA, Sham JS, Shimoda TH, and Sylvester JT. L-type Ca2+ channels, resting [Ca2+]i, and ET-1-induced responses in chronically hypoxic pulmonary myocytes. *Am J Physiol Lung Cell Mol Physiol* 279: L884–L894, 2000.

133. Singh DK, Kumar D, Siddiqui Z, Basu SK, Kumar V, and Rao KV. The strength of receptor signaling is centrally controlled through a cooperative loop between Ca2+ and an oxidant signal. *Cell* 121: 281–293, 2005.
134. Somlyo AP and Somlyo AV. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 83: 1325–1358, 2003.
135. Srinivasan R, Forman S, Quinlan RA, Ohanian J, and Ohanian V. Regulation of contractility by Hsp27 and Hic-5 in rat mesenteric small arteries. *Am J Physiol Heart Circ Physiol* 294: H961–H969, 2008.
136. Steinberg SF. Structural basis of protein kinase C isoform function. *Physiol Rev* 88: 1341–1378, 2008.
137. Su J, Muranjan M, and Sap J. Receptor protein tyrosine phosphatase alpha activates Src-family kinases and controls integrin-mediated responses in fibroblasts. *Curr Biol* 9: 505–511, 1999.
138. Sun G, Ramdas L, Wang W, Vinci J, McMurray J, and Budde RJ. Effect of autophosphorylation on the catalytic and regulatory properties of protein tyrosine kinase Src. *Arch Biochem Biophys* 397: 11–17, 2002.
139. Tabet F, Schiffrin EL, and Touyz RM. Mitogen-activated protein kinase activation by hydrogen peroxide is mediated through tyrosine kinase-dependent, protein kinase C-independent pathways in vascular smooth muscle cells: upregulation in spontaneously hypertensive rats. *J Hypertens* 23: 2005–2012, 2005.
140. Taher MM, Garcia JG, and Natarajan V. Hydroperoxide-induced diacylglycerol formation and protein kinase C activation in vascular endothelial cells. *Arch Biochem Biophys* 303: 260–266, 1993.
141. Tang H, Hao Q, Rutherford SA, Low B, and Zhao ZJ. Inactivation of SRC family tyrosine kinases by reactive oxygen species *in vivo*. *J Biol Chem* 280: 23918–23925, 2005.
142. Thakali K, Davenport L, Fink GD, and Watts SW. Cyclooxygenase, p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase MAPK, Rho kinase, and Src mediate hydrogen peroxide-induced contraction of rat thoracic aorta and vena cava. *J Pharmacol Exp Ther* 320: 236–243, 2007.
143. Tonks NK. Redox redux: revisiting PTPs and the control of cell signaling. *Cell* 121: 667–670, 2005.
144. Touyz RM, Yao G, and Schiffrin EL. c-Src induces phosphorylation and translocation of p47phox: role in superoxide generation by angiotensin II in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 23: 981–987, 2003.
145. Ungvari Z, Csiszar A, Huang A, Kaminski PM, Wolin MS, and Koller A. High pressure induces superoxide production in isolated arteries via protein kinase C-dependent activation of NAD(P)H oxidase. *Circulation* 108: 1253–1258, 2003.
146. Ushio-Fukai M. Localizing NADPH oxidase-derived ROS. *Sci STKE* 2006: re8, 2006.
147. Ushio-Fukai M, Griendling KK, Becker PL, Hilenski L, Halleran S, and Alexander RW. Epidermal growth factor receptor transactivation by angiotensin II requires reactive oxygen species in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 21: 489–495, 2001.
148. Wang QS, Zheng YM, Dong L, Ho YS, Guo Z, and Wang YX. Role of mitochondrial reactive oxygen species in hypoxia-dependent increase in intracellular calcium in pulmonary artery myocytes. *Free Radic Biol Med* 42: 642–653, 2007.
149. Wang T, Chiang ET, Moreno-Vinasco L, Lang GD, Pendyala S, Samet JM, *et al.* Particulate matter disrupts human lung endothelial barrier integrity via ROS- and p38 MAPK-dependent pathways. *Am J Respir Cell Mol Biol* 42: 442–449, 2010.
150. Ward JP. A twist in the tail: synergism between mitochondria and NADPH oxidase in the hypoxia-induced elevation of reactive oxygen species in pulmonary artery. *Free Radic Biol Med* 45: 1220–1222, 2008.
151. Ward JP. Point: Hypoxic pulmonary vasoconstriction is mediated by increased production of reactive oxygen species. *J Appl Physiol* 101: 993–995, 2006.
152. Ward JP, Knock GA, Snetkov VA, and Aaronson PI. Protein kinases in vascular smooth muscle tone-role in the pulmonary vasculature and hypoxic pulmonary vasoconstriction. *Pharmacol Ther* 104: 207–231, 2004.
153. Watanabe H. Rho-kinase activation in patients with pulmonary arterial hypertension. *Circ J* 73: 1597–1598, 2009.
154. Waypa GB, Chandel NS, and Schumacker PT. Model for hypoxic pulmonary vasoconstriction involving mitochondrial oxygen sensing. *Circ Res* 88: 1259–1266, 2001.
155. Waypa GB, Marks JD, Guzy R, Mungai PT, Schriewer J, Dokic D, *et al.* Hypoxia triggers subcellular compartmental redox signaling in vascular smooth muscle cells. *Circ Res* 106: 526–535, 2010.
156. Waypa GB, Marks JD, Mack MM, Boriboun C, Mungai PT, and Schumacker PT. Mitochondrial reactive oxygen species trigger calcium increases during hypoxia in pulmonary arterial myocytes. *Circ Res* 91: 719–726, 2002.
157. Wedgwood S, Dettman RW, and Black SM. ET-1 stimulates pulmonary arterial smooth muscle cell proliferation via induction of reactive oxygen species. *Am J Physiol Lung Cell Mol Physiol* 281: L1058–L1067, 2001.
158. Weir EK, Lopez-Barneo J, Buckler KJ, and Archer SL. Acute oxygen-sensing mechanisms. *N Engl J Med* 353: 2042–2055, 2005.
159. Weissmann N, Voswinckel R, Hardebusch T, Rosseau S, Ghofrani HA, Schermuly R, *et al.* Evidence for a role of protein kinase C in hypoxic pulmonary vasoconstriction. *Am J Physiol* 276: L90–L95, 1999.
160. Wilson DP, Susnjar M, Kiss E, Sutherland C, and Walsh MP. Thromboxane A2-induced contraction of rat caudal arterial smooth muscle involves activation of Ca2+ entry and Ca2+ sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. *Biochem J* 389: 763–774, 2005.
161. Wojciak-Stothard B, Tsang LY, and Haworth SG. Rac and Rho play opposing roles in the regulation of hypoxia/reoxygenation-induced permeability changes in pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 288: L749–L760, 2005.
162. Wolin MS. Reactive oxygen species and the control of vascular function. *Am J Physiol Heart Circ Physiol* 296: H539–H549, 2009.
163. Yamboliev IA, Hedges JC, Mutnick JL, Adam LP, and Gerthoffer WT. Evidence for modulation of smooth muscle force by the p38 MAP kinase/HSP27 pathway. *Am J Physiol Heart Circ Physiol* 278: H1899–H1907, 2000.
164. Yan SF, Harja E, Andrassy M, Fujita T, and Schmidt AM. Protein kinase C beta/early growth response-1 pathway: a key player in ischemia, atherosclerosis, and restenosis. *J Am Coll Cardiol* 48(9 Suppl 1): A47–A55, 2006.

165. Yang ZW, Zheng T, Wang J, Zhang A, Altura BT, and Altura BM. Hydrogen peroxide induces contraction and raises [Ca2+]i in canine cerebral arterial smooth muscle: participation of cellular signaling pathways. *Naunyn Schmiedebergs Arch Pharmacol* 360: 646–653, 1999.
166. Zemlickova E, Johannes FJ, Aitken A, and Dubois T. Association of CPI-17 with protein kinase C and casein kinase I. *Biochem Biophys Res Commun* 316: 39–47, 2004.
167. Zhang J, Jin N, Liu Y, and Rhoades RA. Hydrogen peroxide stimulates extracellular signal-regulated protein kinases in pulmonary arterial smooth muscle cells. *Am J Respir Cell Mol Biol* 19: 324–332, 1998.
168. Zhang J, Xing D, and Gao X. Low-power laser irradiation activates Src tyrosine kinase through reactive oxygen species-mediated signaling pathway. *J Cell Physiol* 217: 518–528, 2008.
169. Zhao Y and Davis HW. Hydrogen peroxide-induced cytoskeletal rearrangement in cultured pulmonary endothelial cells. *J Cell Physiol* 174: 370–379, 1998.

Address correspondence to:
Dr. Greg A. Knock
Division of Asthma, Allergy, and Lung Biology
King's College London
Room 3.20, Franklin Wilkins Building
Stamford St.
London SE1 9NH
United Kingdom

E-mail: greg.knock@kcl.ac.uk

Date of first submission to ARS Central, August 28, 2010; date of acceptance, September 19, 2010.

Abbreviations Used

Ang II = angiotensin II
Cat = catalase
COX = cyclooxygenase
DAG = diacylglycerol
EGF-R = epidermal growth factor receptor
ERK = extracellular signal-regulated kinase
ET-1 = endothelin-1
ETC = electron transport chain
GEF = guanine exchange factor
Grb2 = growth-factor receptor binding protein-2
H_2O_2 = hydrogen peroxide
HPV = hypoxic pulmonary vasoconstriction
HSP27 = heat-shock protein 27
LDL = low-density lipoprotein
LOX = lipoxygenase
MAPKs = mitogen-activated protein kinases
MASMC = mesenteric artery smooth muscle cell
MLCK = myosin light-chain kinase
MLCP = myosin light-chain phosphatase
MYPT-1 = myosin phosphatase targeting subunit
NO = nitric oxide
NOX = NADPH oxidase
PAEC = pulmonary artery endothelial cell
PASMC = pulmonary artery smooth muscle cell
PKC = protein kinase C
PLC = phospholipase C
PS = pseudo-substrate
PTP = protein tyrosine phosphatase
ROK = Rho-kinase
ROS = reactive oxygen species
SOD = superoxide dismutase
SoS = son-of-sevenless
srcFK = src-family kinases

This article has been cited by:

1. S. Mejrbi, R. Lakhdar, L. Chaieb, J. Ben Chibani, A. Miled, A. Kassab. 2012. L'impact de l'équilibre glycémique sur le statut oxydant–antioxydant dans un échantillon de diabétiques de type 2 tunisiens. *Immuno-analyse & Biologie Spécialisée* . [CrossRef]
2. Aurelie Nguyen Dinh Cat , Augusto C. Montezano , Dylan Burger , Rhian M. Touyz . Angiotensin II, NADPH Oxidase, and Redox Signaling in the Vasculature. *Antioxidants & Redox Signaling*, ahead of print. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
3. David I. Pattison, Aldwin Suryo Rahmanto, Michael J. Davies. 2012. Photo-oxidation of proteins. *Photochemical & Photobiological Sciences* . [CrossRef]
4. Wei Wang, Zelieann R. Craig, Mallikarjuna S. Basavarajappa, Rupesh K. Gupta, Jodi A. Flaws. 2011. Di (2-ethylhexyl) phthalate inhibits growth of mouse ovarian antral follicles through an oxidative stress pathway. *Toxicology and Applied Pharmacology* . [CrossRef]