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Forum Review

Mechanisms of Cell Signaling by Nitric Oxide and Peroxynitrite: From Mitochondria to MAP Kinases

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ABSTRACT

Many of the biological and pathological effects of nitric oxide (NO) are mediated through cell signaling pathways that are initiated by NO reacting with metalloproteins. More recently, it has been recognized that the reaction of NO with free radicals such as superoxide and the lipid peroxyl radical also has the potential to modulate redox signaling. Although it is clear that NO can exert both cytotoxic and cytoprotective actions, the focus of this overview are those reactions that could lead to protection of the cell against oxidative stress in the vasculature. This will include the induction of antioxidant defenses such as glutathione, activation of mitogen-activated protein kinases in response to blood flow, and modulation of mitochondrial function and its impact on apoptosis. Models are presented that show the increased synthesis of glutathione in response to shear stress and inhibition of cytochrome *c* release from mitochondria. It appears that in the vasculature NO-dependent signaling pathways are of three types: (i) those involving NO itself, leading to modulation of mitochondrial respiration and soluble guanylate cyclase; (ii) those that involve *S*-nitrosation, including inhibition of caspases; and (iii) autocrine signaling that involves the intracellular formation of peroxynitrite and the activation of the mitogen-activated protein kinases. Taken together, NO plays a major role in the modulation of redox cell signaling through a number of distinct pathways in a cellular setting. Antioxid. Redox Signal. 3, 215–229.

INTRODUCTION

IN THE CELL, the concept that oxidative stress, or more recently nitrosative stress, contributes to both normal physiology and the development of human disease is well established particularly in the fields related to the pathology of atherosclerosis (5, 90). At a molecular level, mechanisms for the antioxidant action of a molecule can include direct reactions with oxidants as scavengers and more complex effects on the regulation of antioxidant enzymes and intracellular defenses (2, 106). This diversity of action, however, unusual for one molecule, and in this respect nitric oxide (NO) is particularly interesting. The focus here will be the antioxidant effects of NO and the mechanisms that may underlie these responses with an emphasis on the vascular system. Although it is clear that NO can exert cytotoxic effects especially in combination with superoxide (O_2^-) yielding peroxynitrite

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($ONOO^-$), this may also serve an autocrine signaling function (4, 20, 39, 41, 46, 78, 90).

In addition to the direct reaction with free radical species both intra- and extracellularly, NO can exert important biological effects through its activation of specific cell-signaling pathways. An important example that we will discuss in some detail is the activation of endothelial NO synthase (eNOS) by shear stress, the force exerted on the endothelium in response to laminar blood flow (31). NO-dependent signal-transduction pathways are now known to control the concentrations of cellular antioxidants, modulate cell proliferation or apoptosis, and regulate the response of cells to inflammation (23, 84). One well understood example is the role soluble guanylate cyclase (sGC) plays in modulating inflammation and vascular function, but these aspects have already been discussed in a number of excellent reviews (22, 47). In this article, some emerging concepts will be selected that highlight each of the potential mechanisms that could contribute to an antioxidant effect of NO. The interaction of NO with metals, particularly iron, will be discussed in the context of the emerging role of various forms of the NO–hemoglobin complex. Also the effect of NO on mitochondrial respiration will be described, as well as the effects of NO on cytochrome *c* release and its potential importance for apoptosis. More indirect mechanisms in which NO can regulate transcriptional events related to control of oxidative stress will be described, and this will be placed in the context of the response of the endothelium to shear stress.

REACTIONS OF NO AND REACTIVE NITROGEN SPECIES (RNS) THAT MODIFY PROTEINS

The biochemistry of NO is dominated by the generally rapid and facile reaction with other free radicals and the reversible binding to metalloproteins. These two types of reaction are the routes through which this free radical has the potential to reversibly modify specific amino acids on proteins. In turn, this provides a mechanism for the modulation of enzymatic pathways whether central to metabolism or involved in cell signaling. The specific pathways that form the focus of most current research in this area are illustrated in Fig. 1A. The reactions for which we have some direct information in biological systems include the binding of NO to Fe in metalloproteins, and the secondary reactions of NO with reactive oxygen species, and indeed oxygen itself, to form stable and reversible adducts with proteins (22, 29, 47, 48, 83, 96, 97). A modification that is currently favored in many circles as a route for the modulation of thiol-containing proteins is the *S*-nitrosation reaction (25, 63, 67, 83). An interesting series of experiments suggest that modification

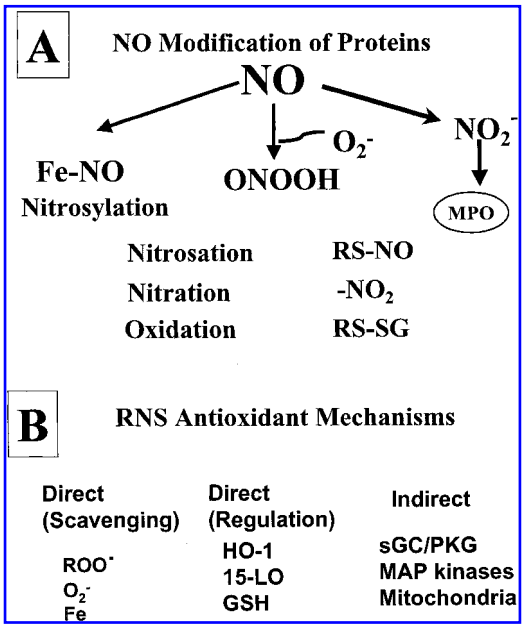

FIG. 1. NO-dependent modifications of biomolecules and antioxidant pathways. (**A**) NO reacts rapidly with O_2^- forming $ONOO^-$. This leads to oxidation, nitrosation (addition of NO), or nitration (addition of NO_2) of biomolecules. Recently, it has also been shown that the enzyme myeloperoxidase (MPO) can cause nitration reactions using nitrite (NO_2^-) as a substrate. The oxidation reactions (*e.g.*, oxidation of glutathione) occur at the highest chemical yield, but the nitration (*e.g.*, formation of nitrotyrosine) or nitrosation (*e.g.*, formation of *S*-nitrosoglutathione) reactions are also biologically significant because they can alter cell signaling and generate NO donors. (**B**) NO and its oxidation products can work as antioxidants either reacting directly with peroxides ($ROO^•$), O_2^- or iron or through regulation of antioxidant or prooxidant pathways, for example, by up-regulating glutathione (GSH) or heme oxygenase-1 (HO-1) or inhibiting 15-lipoxygenase (15-LO). NO can also work as an antioxidant indirectly through activation of soluble guanylate cyclase (sGC/PKG), mitogen-activated protein (MAP) kinase pathways or mitochondrial function.

of the caspase enzymes that play an essential role in apoptosis can be modified by this mechanism (25, 63, 67). The oxidation reactions mediated by RNS were among the first to be identified in a biological setting, but it remains uncertain whether, for example, tyrosine nitration plays a role in cell signaling. Given the different mechanisms through which NO could exert biological effects, it is not surprising that NO can act as an antioxidant through diverse routes. The general categories are summarized in Fig. 1B. They include direct scavenging of radical species, such as O_2^- or lipid peroxyl radicals (41, 46, 81, 82, 90). The caveat here is that it is important to know what responses the products of the reaction of NO with the free radical in question elicit in a cellular setting. The second category we have defined is the direct modification of antioxidant pathways in the cell. In the third category we have grouped those mechanisms that appear to involve direct modulation of signal-transduction pathways with a potential outcome that can result in increased antioxidant protection.

In the following sections we will describe some specific examples to illustrate the complexity of the signaling pathways that can be influenced by RNS.

NITROSYLATION OF METALLOPROTEINS

Reactions between metalloproteins and NO are important in mediating many of the biological effects of this free radical (19). Most work has been focused around elucidating the reactions between NO and iron-containing (both heme and non-heme) proteins, although interactions with copper in metalloproteins have also been implicated (101).

The affinity of NO for heme iron, predominantly in the ferrous oxidation state (Fe^{2+}), is well established and is a reaction known as nitrosylation. This is distinct from *S*-nitrosation reactions, where an NO^+ group is linked to a thiol. Binding of NO to ferrous iron can displace the metal from the planar porphyrin ring of the heme protein and so change the activity of enzymes (19, 102, 109). The classical example is activation of SGC leading to the production of cyclic GMP (47, 52, 73). This process is well documented as a mechanism that contributes to NO-dependent regulation of vascular tone and inhibition of platelet aggregation (47, 73). Similar reactions occurs between NO and deoxygenated hemoglobin and the product of this reaction, nitrosylhemoglobin, has been measured *in vivo* (37, 55, 56, 100). Interestingly, formation of nitrosylhemoglobin promotes transition of the protein to the low oxygen affinity form, and has therefore been suggested to stimulate oxygen delivery (55, 109). NO reactions with heme proteins are not limited to the ferrous oxidation state. Rapid reactions between ferryl heme proteins have been reported and implicated in the mechanisms by which NO regulates the activity of enzymes that redox cycle between different heme oxidation states (1, 12, 13, 40). Catalase myeloperoxidase are important examples of this (1, 12). These reactions are likely to be important in the antioxidant/antiinflammatory functions of NO, by preventing toxicity mediated by ferryl heme proteins (40). In this reaction, NO is thought to reduce the ferryl heme to the corresponding ferric forms (1, 12, 13, 40). It should also be noted that NO will also reduce ferric heme iron, albeit at a slow rate that may not contribute significantly in a physiological setting (19).

Rapid reactions between NO and oxygenated heme proteins also occur and with hemoglobin lead to the production of nitrate and ferric or methemoglobin (metHb) (19, 28). This reaction may represent a major metabolic route for NO and/or nitrite. In recent studies, a complex series of reactions have been put forward in which NO interactions with the heme group of hemoglobin result in the formation of *S*-nitrosohemoglobin (SNOHb) (43, 44, 51, 98). This serves to highlight the possibility that heme proteins regulate different redox states of NO and hence its biological action (97). SNOHb represents an intriguing example in this context. This derivative of hemoglobin in which NO is bound to a specific cysteine residue in the β-chain modulates the oxygen binding properties of hemoglobin (9, 83). More controversial is the proposal that SNOHb mediates blood flow *in vivo* (51, 98). These concepts derive primarily from observations that demonstrate the ability of SNOHb to mediate vessel

relaxation in an oxygen-sensitive manner (51, 69). However, other studies indicate that the oxygen sensitivity is not at the level of the hemoglobin protein, and that in humans a significant arterial–venous gradient in SNOHb does not exist (38, 83, 107). A further example of a nitrosylation reaction is the reversible binding of cytochrome *c* oxidase by NO at the oxygen binding site (17). The implications of this interaction will be discussed in the next section.

NO AND MITOCHONDRIA

A potentially important site for NO signaling in the cell that could lead to antioxidant effects is the mitochondrion. At present, little is known of this interaction and its implications for control of oxidative stress in the cell. As the mitochondrion has long been thought of as a major site for O_2^- formation, this is an important issue. A perspective that is gaining support is that under some circumstances mitochondrially generated O_2^- may play a role in cellular signaling (7, 61, 79), particularly in the activation of stress-related signaling pathways, such as mitogen-activated protein (MAP) kinases, which are involved in transcriptional regulation of many proteins, including antioxidant enzymes. An aspect of this process that has received little attention is the inhibition of mitochondrial respiration by NO. NO can inhibit cytochrome *c* oxidase reversibly (17), and other parts of the respiratory chain both reversibly and irreversibly (18, 87). It has been suggested that such inhibition serves to elevate mitochondrial O_2^- generation (86), and this raises the possibility that mitochondria may transduce a nitrosative signal into an oxidative one. It is important to note that although O_2^- is the most likely initial product from mitochondrial respiration at complexes I, II, and III, the high concentration of manganese superoxide dismutase (SOD) associated with the inner mitochondrial membrane results in the release of hydrogen peroxide from the organelle.

A potential site for modulation of mitochondrial O_2^- generation is the proton permeability of the mitochondrial inner membrane. It is proposed that the dissipation of redox energy that occurs via this "proton leak" serves to decrease O_2^- generation by allowing continued O_2 consumption when ATP demand is low (94, 95). This is consistent with observations that endotoxin (lipopolysaccharide) treatment, which up-regulates inducible NO synthase (108), also up-regulates mitochondrial uncoupling proteins (110), which are putative mediators of the proton leak. This would serve an antioxidant function by coordinately minimizing O_2^- production in the presence of NO, thereby limiting $ONOO^-$ generation. In support of this concept, it has also been shown that $ONOO^-$ increases brain mitochondrial proton leak (10), which may be part of a feedback loop mechanism to prevent further $ONOO^-$ formation at the mitochondrial level.

This is a promising area for developing the concept that NO interactions with the mitochondria can control cytosolic signaling events (86). Additional control mechanisms include regulation of the mitochondrial O_2^- generation by modifying electron flux through different segments of the respiratory chain (electrons) and the recent observation that $ONOO^-$ can modulate the activity of glycolysis and the Krebs cycle (16, 24).

The inhibition of apoptosis by NO through interactions with mitochondria is a new aspect of mitochondrial biology that has previously received little attention. Certain apoptotic stimuli cause mitochondria to release their cytochrome *c*, which then activates caspases, the molecular executors of apoptotic signaling (64) (Fig. 2). We recently showed that NO inhibits mitochondrial cytochrome *c* release via a mechanism involving inhibition of Ca^{2+} accumulation (Fig. 2B). In addition, NO appeared to alter the relationship between mitochondrial swelling and cytochrome *c* release, such that a lower threshold of swelling was required for cytochrome *c* release to occur (Fig. 2C) (11). It has been suggested that cytochrome *c* loss may be responsible for the burst of mitochondrial O_2^- generation associated with apoptosis (15), and although the role of this oxidative burst in apoptotic signaling has yet to be identified, NO inhibition of cytochrome *c* release would impact on this. Clearly, the potential exists for a number of intracellular signaling events, particularly those associated with oxidative stress and antioxidant regulation, to be mediated by the

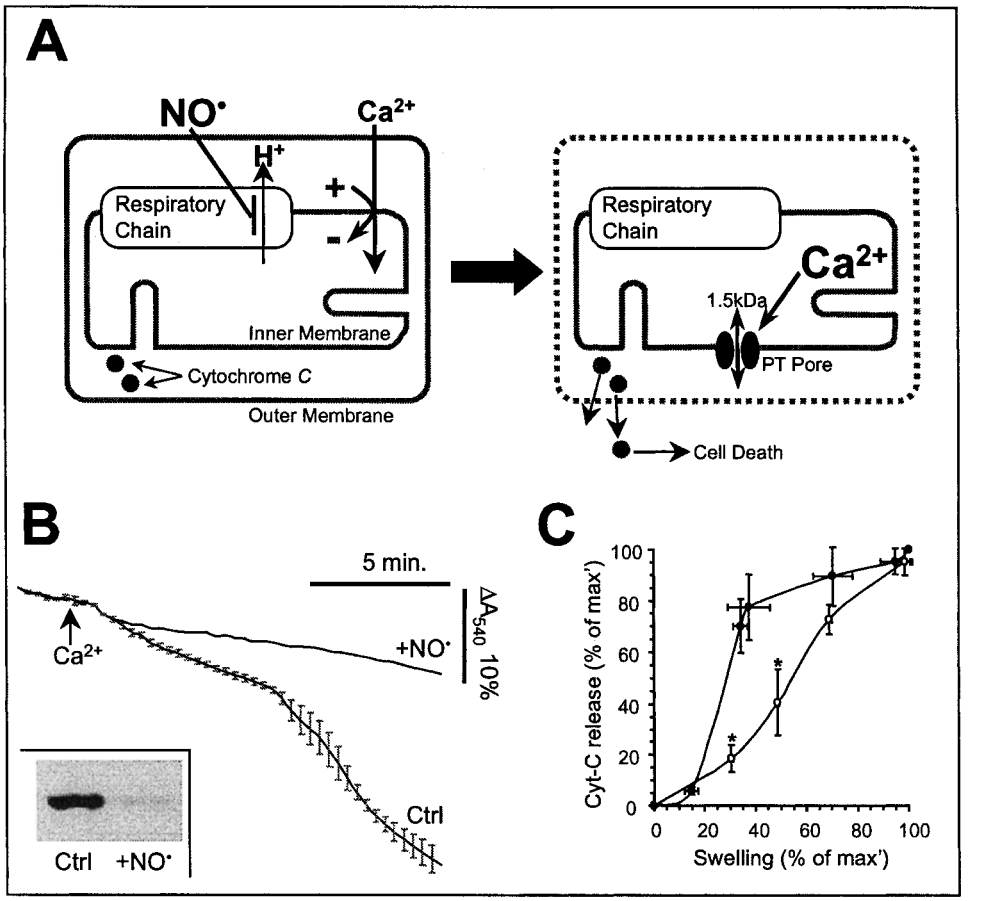

FIG. 2. The effects of NO on mitochondrial cytochrome *c* release. (**A**) Schematic showing mitochondrial cytochrome *c* release. Ca^{2+} accumulation is driven electrochemically by a protein gradient (+ and − signs) generated by the respiratory chain. High intramitochondrial [Ca^{2+}] triggers opening of the permeability transition (PT) pore. This is linked to the release of cytochrome *c* (circles) via an unknown mechanism possibly involving outer membrane rupture and swelling. NO inhibits respiration, and therefore inhibits the driving force for Ca^{2+} accumulation, preventing PT pore opening and cytochrome *c* release. This is shown experimentally in **B**. Mitochondrial PT pore opening was induced by addition of Ca^{2+} and monitored via light scattering (caused by mitochondrial swelling) at 540 nm (11). In the presence of an NO donor (spermine NONOate, 1.1 m*M*), both swelling and cytochrome *c* release (**inset:** western blot of mitochondrial supernatants) were inhibited. (**C**) Effects of NO on the relationship between swelling and cytochrome *c* release. Parameters were measured as in B, in the absence (○) and presence (●) of spermine NONOate. Results are means ± SEM of at least five independent experiments. $^*p < 0.05$ between experimental groups.

complex interaction between NO and mitochondria. Further studies in both intact cells and isolated mitochondria will aid in elucidating the precise role that this interaction plays in the cytoprotective and antioxidant functions of NO.

TRANSCRIPTIONAL REGULATION AND MODULATION OF ANTIOXIDANT SYSTEMS BY RNS

Apart from direct antioxidant functions associated with scavenging of reactive species, NO may provide cytoprotection through upregulation of antioxidant systems in the cell. NO induces the expression of antioxidant enzymes manganese and copper-zinc SODs and heme oxygenase-1 (HO-1) (76, 91, 92). Furthermore, NO increases intracellular glutathione (GSH) concentration by increasing cellular cystine uptake and γ-glutamylcysteine synthetase (GCS), the rate-limiting enzyme of GSH synthesis (62, 70, 71). Thus, both determinants of GSH synthesis, the supply of limiting substrate cysteine and the activity of GCS, are increased concomitantly. NO appears to be an important

regulator of GSH synthesis also *in vivo*, because GCS activity is down-regulated by 50% by chronic NO synthase inhibition in rats (60).

In vascular smooth muscle cells, NO up-regulates the mRNA of both the catalytic (heavy) and regulatory (light) subunits of GCS (70). The pathways involved in the NO-dependent signaling for GCS are not known, but these appear to be independent of cyclic GMP (70, 71). It is also unknown whether the increase in GCS mRNA is through increased transcription of GCS genes or enhanced stability of the mRNA. Recently, translation-independent stabilization of HO-1 mRNA has been shown to occur upon exposure to NO donors (9). A potential candidate to mediate this effect is iron regulatory protein-1, which regulates posttranscriptionally the synthesis of several proteins important for iron and oxygen metabolism and is known to be activated by NO through either binding to the iron-sulfur cluster or liberation of cellular Fe (105). Enhancement of GCS light and heavy subunit stability through a similar mechanism is also a possibility that needs to be investigated in detail.

In addition to increased mRNA stability, GCS expression may be regulated through enhanced transcription of the heavy and light subunit genes. The promoter regions of both genes contain a number of putative transcription factor binding sites including activator protein-1, Sp-1, antioxidant responsive element/electrophile responsive element, and nuclear factor κB (NF-κB), which are potential targets for NO-dependent regulation (72, 77). Depending on the concentration of NO as well as the transcription factors involved, these undergo either increased or decreased binding upon exposure to NO. For example, low concentrations of NO are thought to activate NF-κB, possibly through *S*-nitrosation of the upstream molecule $p21^{ras}$ and subsequent activation of IκB kinases (50, 59). However, high concentrations inhibit NF-κB due to increased stability IκB kinases (50, 85). Whereas Sp-1 has been recently shown to be activated by NO through a cyclic GMP-independent mechanism (104), high concentrations of NO inhibit the binding of Sp-1 and other zinc-finger transcription factors (57). Also direct inhibition of DNA binding through disruption of other, yet uncharacterized metal-binding thiol transcription factors similar to yeast transcription factor Ace1 is a possibility (93).

The mediators involved in the NO-dependent GCS induction may also be secondary oxidants, such as $ONOO^-$. In this respect, a recent report showing increased GSH levels upon exposure to 3-morpholinosydnonimine, a $ONOO^-$ donor, is of particular interest (14). Although the authors state that this increase is mediated through increased cystine uptake, a concomitant GCS induction is not excluded. $ONOO^-$ has also been suggested to be involved in the induction of HO-1 by NO, and $ONOO^-$ itself is able to induce this enzyme in endothelial cells (32, 33). Certain cell types are sensitive to the cytotoxicity of $ONOO^-$, but endothelial cells are known to be more resistant, although high concentrations are known to be proapoptotic also in this cell type (33, 58). However, if $ONOO^-$ mediates GCS induction by NO donors, it is likely to serve a signaling function as the NO fluxes needed for GCS induction (1–3 n*M*/s) are far lower than those concentrations that are cytotoxic (71).

Intracellular GSH concentration appears to be a critical determinant in many functions of NO in the cell. Depletion of intracellular GSH by GCS inhibitor buthionine sulfoximine attenuates vascular smooth muscle cell relaxation and subsequent vasodilation caused by nitroglycerin (6). Moreover, GSH depletion enhances the cytotoxicity of NO and $ONOO^-$, indicating the important role of GSH in the detoxification of RNS (66, 103). In purified enzyme preparations of inducible and neuronal NO synthase, GSH is needed for full enzyme activity (45, 99). Furthermore, endothelial NO synthesis is impaired in GSH-depleted endothelial cells, and conversely, enhanced by a thiol antioxidant and GSH precursor *N*-acetylcysteine (36, 88). Thus, there appears to be a close interaction between NO and GSH metabolism, and GSH not only protects against RNS-mediated toxicity, but also is needed for NO synthesis.

$ONOO^-$ AND CELL SIGNALING

Peroxynitrite at high concentrations can cause a wide spectrum of oxidation and nitra-

tion reactions that can lead to cell death (3). However, even in the earliest studies, exposure of cellular systems to the lower more physiological, rather than pathological, concentrations appeared to be having more specific and even protective effects. For example, exposure of $ONOO^-$ to isolated aortic vessels, perfused hearts, and platelets resulted in the formation of NO donors through formation of nitrated thiols or alcohol groups (68, 74, 75). Mechanisms are still be elucidated as these studies are confirmed and extended, but they do raise the tantalizing possibility that $ONOO^-$ can function as a cell-signaling molecule. For example, a recent study associated the cardioprotective effect of $ONOO^-$ at low concentrations of 0.2–2 μM with decreased P-selectin expression and preservation of endothelial cell function (80).

Initially, this concept has met some resistance, because $ONOO^-$ is reactive and chemically unstable in molecular terms, but a biological half-life of seconds does not preclude a role as a cell-signaling molecule. This occurs because of the dominance of the nitration reactions over oxidation in biological systems exposed to RNS such as $ONOO^-$ and the effect of carbon dioxide in favoring nitration reactions over oxidation (42, 65). Thus, the short-lived effects of $ONOO^-$ generated within the cell in a specific domain may lead to specific modification and hence control of selected signal-transduction pathways.

Direct addition of $ONOO^-$ to cells can result in activation of the MAP kinases, although little selectivity was evident in the activation of the three major classes of these proteins. In a series of studies, this was also examined in endothelial cells subject to shear stress and exposed to a bolus concentration of $ONOO^-$. Shear stress induces the formation of both NO and O_2^- within the cell and activates the MAP kinases extracellular signal-regulated protein kinase (ERK) and N-terminal c-Jun kinase (JNK) (39). A typical result is shown in Fig. 3, and it further indicates that the physiological generation of both NO and O_2^- in this context does not lead to activation of ERK. NO alone at these concentrations does not activate JNK (or ERK), and the hypothesis was tested that the cogeneration of O_2^- was contributing to this effect. These data are shown in Fig. 3C and show that overexpression of SOD, but not catalase, inhibits JNK activation. The addition of bolus $ONOO^-$ to the cells under a static condition resulted in activation of JNK to a level similar to that found with shear stress. The bolus concentration of $ONOO^-$ was calculated to be at least 50 times greater than that produced in response to shear stress. In support of this finding, staining with the nitrotyrosine antibody of the cells that were subjected to shear stress showed a significant (twofold) increase (Fig. 3E). The staining for cells exposed to bolus $ONOO^-$, on the other hand (Fig. 3F), was far more extensive and particularly prominent at the cell surface.

This is consistent with the formation of $ONOO^-$ in an intracellular cell-signaling domain composed of multiprotein complexes. This is precedented in NO signaling. For example, in the case of eNOS the association of the enzyme with subcellular structures known as caveolae modulates NO production in response to a wide range of agonists (30). If reactive molecules such as $ONOO^-$ play a role in signal transduction, having the "receptor" closely located to the site of formation may be a mechanism to endow specificity to the signaling cascade and may also indicate that this is an autocrine signaling mechanism.

In the previous section, the induction of GSH synthesis by NO and $ONOO^-$ was discussed. Physiologically, shear stress is a regulator of NO production and is associated with the increased production of eNOS and antioxidant enzymes such as the SODs (26). Therefore, we hypothesized that shear stress could increase GSH in endothelial cells through NO formation. To test this idea, endothelial cells were exposed to shear stress (5 dyn/cm^2), and cell lysates were used to determine total GSH content and GSH oxidation over the first hour (Fig. 4). Within the first minutes of shear stress, a highly significant oxidation of GSH occurs, but without a change in the total GSH. After a period of 12–16 h, a 1.5–2-fold increase in GSH has occurred.

This rapid oxidation of GSH stimulated by shear stress is accompanied by the immediate

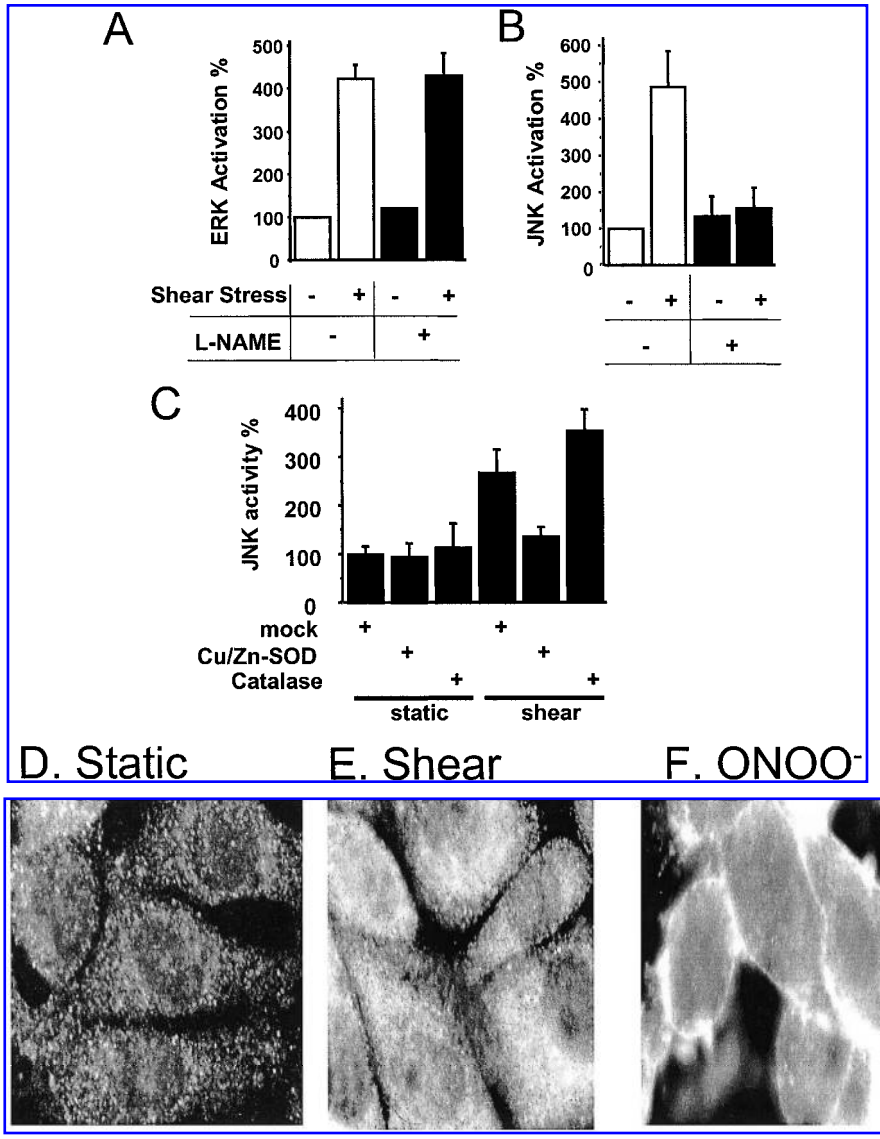

FIG. 3. Activation of MAP kinase pathways by shear stress (A and B): Inhibition of NO production prevents shear-dependent activation of JNK, but not ERK. BAEC (bovine aortic endothelial cells) were incubated without or with 3 m*M* N^{ω}-nitro-L-arginine methyl ester (L-NAME) for 10 min. Cells were then subjected to static control or 10 dyn/cm^2 of shear stress for 5 min (A) or for 1 h (B). Activity of ERK was determined with a phospho-ERK (pERK) antibody by western blot analysis (A) and JNK activity by the phosphorylation of GST-cJun (B) and subsequent separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (39). The data are expressed as means ± SEM (n = 3). (**C**) Scavenging O_2^-, but not H_2O_2, prevented shear-dependent activation of JNK. Cells were transiently cotransfected with a vector encoding for Cu/Zn-SOD, catalase, or empty vector control (pcDNA) along with hemagglutinin-tagged JNK1. Two days after transfection, cells were subjected to static control or shear stress and the JNK activity was measured. Data are expressed as means ± SEM (n = 3). (**D**, **E**, and **F**): Shear stress increases the nitrotyrosine staining in endothelial cells. BAEC were exposed to static condition (D) or to shear stress (10 dyn/cm^2; E) for 1 h or to authentic $ONOO^-$ (F) for a few seconds. BAEC were then incubated with a nitrotyrosine antibody followed by a Cy3-conjugated goat anti-rabbit antibody. Washed cells were then mounted with Slow-Fade and examined by fluorescence microscopy (39). Representative pictures of three to five independent experiments are shown.

production of NO. It is not yet clear how these mechanisms may be coupled to the regulation of gene expression that can lead to enhanced antioxidant capacity within the sheared cell. However, progress has been made in defining some of the early responses to shear stress that could lead to transcriptional regulation of proteins. Current studies aim to define the mechanisms leading to increased GSH synthesis in response to shear stress.

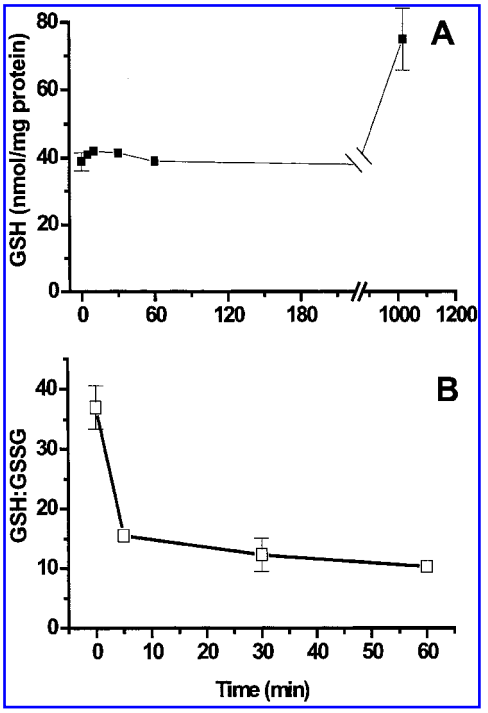

FIG. 4. Effect of shear stress on GSH oxidation and synthesis. Confluent BAEC were exposed to shear (5 dyn/cm^2) for 5 min to 22 h, and the total GSH concentration (**A**) and the GSH:GSSG ratio (**B**) were measured (39, 70). The data for control cells not subjected to shear are also shown (□) and were significantly different from the cell subjected to shear ($p < 0.005$). The data represent the means ± SEM of three to five independent experiments.

THE ROLE OF NO IN REGULATION OF CELL SURVIVAL

As discussed previously, NO has an important role in the regulation of cell death. Whereas certain cell types such as neurons, pancreatic β-cells, and macrophages are exquisitely sensitive to NO-mediated cytotoxicity, others can be protected by NO against toxic effects of various stimuli (23, 53). In the vasculature, NO has been shown to have proapoptotic or antiproliferative effects in vascular smooth muscle cells, whereas in endothelial cells NO proteins against cell death induced by tumor necrosis factor-α, serum starvation, and hydrogen peroxide (23, 35, 49). Also shear stress has been shown to inhibit apoptosis triggered by these stimuli in an NO-dependent manner (26). Apart from previously mentioned inhibition of permeability transition and subsequent cytochrome *c* release from the mitochondrion, NO affects key enzymes involved in the apoptosis cascade through *S*-nitrosation and inhibition of caspase-3, -6, -7, and -8 (23, 25, 63, 67). Other putative mechanisms by which NO inhibits apoptosis include the inhibition of the recruitment of tumor necrosis factor-α associated death domain through reduction of ceramide production, and up-regulation of antiapoptotic proteins heat shock protein-70 and Bcl-2 (21, 53, 89).

More recently, the attention has been focused on the modulation of cytoprotective pathways such as MAP kinase signaling by NO. For example, it has been reported that NO protects against tumor necrosis factor-α-induced apoptosis by inhibiting protein expression of MAP kinase phosphatase-3, followed by prolonged activation of ERK1/2 exerting prosurvival functions, *e.g.*, through up-regulation of Bcl-2 protein (89). Another signaling pathway important to protection against apoptosis is the PI3K-Akt pathway (34). Recent evidence suggests that shear stress activates eNOS through serine phosphorylation via the PI3K-Akt pathway (27). Low levels of NO produced upon stimulation of this pathway could mediate cytoprotection, possibly through the activation of MAP kinase members.

SUMMARY

The hypothesis outlined in Fig. 5 is that NO participates in redox cell signaling by using at least three distinct mechanisms in the vasculature. The finding that RNS such as $ONOO^-$ may play a role as a cell-signaling molecule is not surprising. However, it is possible that these effects may be restricted to the cell type and signaling domain in which the $ONOO^-$ is formed. The formation of NO itself appears to occur in pulses in the endothelium and can diffuse locally, resulting in control of vascular tone. Current thinking is that the vascular smooth muscle cell "senses" the overall NO concentration in the solution and is unable to distinguish NO derived from one endothelial cell versus another. It is this form of NO that we hypothesize is capable of modulating mitochondrial respiration and apoptosis. Finally, a more stable from of NO, *S*-nitrosothiol, is produced by the endothelium and appears to be

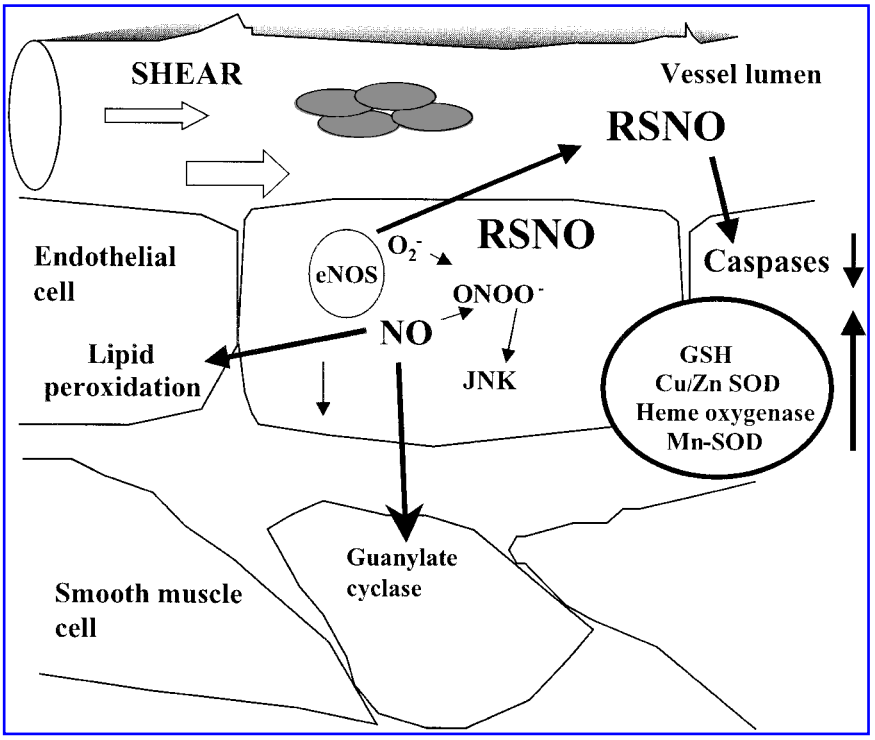

FIG. 5. Three mechanisms of RNS signaling in the vasculature. In this scheme, the effect of shear stress is shown in stimulating NO production through activation of eNOS. Antioxidant mechanisms for the antiatherosclerotic effects of NO are depicted and include inhibition of lipid peroxidation by direct scavenging of lipid radicals and nitrosothiol (RSNO)-dependent inhibition of caspases, which results in protection against apoptotic cell death. The precise molecular identity of the *S*-nitrosothiol remains undefined and is also released from the endothelial cell surface. NO is also shown to increase transcription of a number of genes, including those that regulate intracellular antioxidant systems such as GSH synthesis and the SODs. Diffusion of NO across the endothelial cell membrane results in relaxation of vascular smooth muscle cells. NO is shown reacting with O_2^-, leading to activation of JNK.

in equilibrium with other similar species in the circulation. Rather than controlling vessel tone, this form of NO may modulate other biological processes, such as platelet aggregation (74). Within the cell, the modification of critical thiols on proteins such as Ras and the caspases indicates mechanisms through which the *S*-nitrosothiols can exert a signaling function. Notwithstanding the incomplete nature of our knowledge in this area, it is becoming evident that understanding how these distinct signaling pathways change in response to disease is an interesting question to pursue.

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ABBREVIATIONS

eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated protein kinase; GCS, γ-glutamylcysteine synthetase; GSH, glutathione; HO-1, heme oxygenase-1; JNK, N-terminal c-Jun kinase; MAP kinase, mitogen-activated protein kinase; NF-κB, nuclear factor κB; NO, nitric oxide; O_2^-, superoxide;

$ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; sCG, soluble guanylate cyclase; SNOHb, *S*-nitrosohemoglobin; SOD, superoxide dismutase.

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