Superoxide Fluxes Limit Nitric Oxide-induced Signaling

Received for publication, March 9, 2006, and in revised form, June 1, 2006. Published, JBC Papers in Press, July 7, 2006, DOI 10.1074/jbc.M602242200

Douglas D. Thomas†, Lisa A. Ridnour‡, Michael Graham Espey‡, Sonia Donzelli‡, Stefan Amb§, S. Perwez Hussain§, Curtis C. Harris‡, William DeGraff‡, David D. Roberts‡, James B. Mitchell‡, and David A. Wink‡

From the †Tumor Biology Section, Radiation Biology Branch, the ‡Laboratory of Pathology, and the §Laboratory of Human Carcinogenesis, NCI, National Institutes of Health, Bethesda, Maryland 20892

Indepth, superoxide (O$_2^-$) and nitric oxide (NO) are biologically important signaling molecules. When co-generated, these radicals react rapidly to form powerful oxidizing and nitrating intermediates. Although this reaction was once thought to be solely cytotoxic, herein we demonstrate using MCF7, macrophage, and endothelial cells that when nanomolar levels of NO and O$_2^-$ were produced concomitantly, the effective NO concentration was established by the relative fluxes of these two radicals. Differential regulation of sGC, pERK, HIF-1α, and p53 were used as biological dosimeters for NO concentration. Introduction of intracellular- or extracellular-generated O$_2^-$ during NO generation resulted in a concomitant increase in oxidative intermediates with a decrease in steady-state NO concentrations and a proportional reduction in the levels of sGC, ERK, HIF-1α, and p53 regulation. NO responses were restored by addition of SOD. The intermediates formed from the reactions of NO with O$_2^-$ were non-toxic, did not form 3-nitrotyrosine, nor did they elicit any signal transduction responses. H$_2$O$_2$ in bolus or generated from the dismutation of O$_2^-$ by SOD, was cytotoxic at high concentrations and activated p53 independent of NO. This effect was completely inhibited by catalase, suppressed by NO, and exacerbated by intracellular catalase inhibition. We conclude that the reaction of O$_2^-$ with NO is an important regulatory mechanism, which modulates signaling pathways by limiting steady-state levels of NO and preventing H$_2$O$_2$ formation from O$_2^-$.

Soluble guanylyl cyclase (sGC), extracellular signal-regulated kinases (ERK), hypoxia-inducible factor 1α (HIF-1α), and p53 are post-translationally regulated by nitric oxide (NO). For example, HIF-1α, which is normally limited by proteasome degradation, is stabilized in the presence of NO (1). This leads to translocation to the nucleus, where it activates the transcription of various hypoxia survival genes (2). Similarly, p53 becomes phosphorylated in response to various cellular stresses, including NO metabolites and reactive oxygen species (ROS). p53 phosphorylation can result in cell cycle arrest, which in some cases leads to apoptosis (3). Activation of HIF-1α or p53 therefore has opposing outcomes with respect to cell survival. We have shown previously how distinct threshold NO concentrations are necessary to elicit post-translational regulation of both p53 and HIF-1α. High levels of NO (≥300 nM) cause p53 phosphorylation, whereas intermediate levels (50–300 nM) induce HIF-1α accumulation (1, 4). ERK and sGC are similarly activated by discrete low (10–30 nM) concentrations of NO (5).

It has been proposed that one of the primary reactions of NO in vivo is its reaction with superoxide (O$_2^-$) (6). This diffusion-controlled reaction between NO and O$_2^-$ produces a variety of reactive intermediates that can nitrate, nitrosate, and oxidize many biologically important targets (7–10). Nitrotyrosine formed from this reaction has been associated with functional aberrations in macromolecules. These mechanisms may be important in the etiology of many disease states leading to tissue injury and cell death.

In contrast, a number of studies show that NO is a powerful antioxidant having the ability to abate ROS-mediated oxidative stress (11). The numerous and diametrically opposing outcomes attributed to the reaction of NO with O$_2^-$ have led to much controversy concerning the role of these radicals in physiology and pathophysiology (12–15).

Superoxide dismutase (SOD) reacts with O$_2^-$ in a near diffusion-controlled manner (16). Therefore, cells and tissues with high relative concentrations of this enzyme limit the reaction of O$_2^-$ with NO. This potentially increases local NO concentrations. Reports have shown that in the presence of O$_2^-$, NO-mediated post-translational regulation is abolished and that this can be reversed by the presence of SOD (17).

In cancer biology, the actions of nitric oxide have been shown to have both positive and detrimental effects. Reactive nitrogen species synthase; IFNγ, interferon-γ; LPS, lipopolysaccharide; ANA-1, murine macrophage cells; NBT, nitro blue tetrazolium; p53 S-Pser-15, p53 phosphoserine 15; PBS, phosphate-buffered saline; RNOS, reactive nitrogen oxide species; ROS, reactive oxygen species; O$_2^-$, superoxide; SOD, superoxide dismutase; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; TSP-1, thrombospondin; XO, xanthine oxidase; Sper/NO, (Z)-1-(N-(3-ammoniopropyl)-N-[4-(3-ammoniopropylammonio) butyl]-amino)diazan-1-ium-1,2-diolate.
oxide species have been implicated in both the initiation and promotion of carcinogenesis, yet these same chemical species have been linked to tumor growth suppression (18). Nitric oxide can have beneficial as well as deleterious effects on tumor growth (19, 20). Recent studies have indicated that these contradictory outcomes are linked to different susceptibilities in genotoxicity, cell death, angiogenesis, and metastasis as well as the p53 status of the tumor.

Clinically it has been noted that tumors expressing elevated iNOS and nitrotyrosine indicate a poor prognosis (21). On the other hand, studies using a iNOS transfection xenograft nude mouse model demonstrated an inhibition of tumor growth in a NO concentration-dependent manner (22). Tumor cells transfected with iNOS also resulted in decreased tumorigenesis, which was dependent on both the level of iNOS and the p53 status of the tumors (20, 23, 24). Other studies have demonstrated NOS expression was significantly higher in both primary tumors and lymph nodes than in normal gastric mucosa (25, 26). These and other examples illustrate the dichotomous nature of NO in tumor biology.

We and others (4, 27) have shown that part of these discrepancies could be explained by different concentration dependence of specific signaling targets of NO. Higher NO concentrations activate p53 (>300 nM), while pro-growth mechanisms such as pERK, HIF-1α, and cGC, are activated at lower nanomolar levels (1, 4, 7, 28, 29). Similarly, in endothelial cells, low NO fluxes (<1 nM) lead to increased proliferation and a down-regulation of the anti-angiogenic and tumor suppressor thrombospondin TSP-1 but higher doses induce MKP1 and phosphorylation of p53 (5). Based on these differences in sensitivities of specific signal transduction responses to NO, it follows that any situation resulting in changes in the steady-state NO concentration, such as its reaction with O₂⁻, would have important implications toward cellular behavior.

Although the simple concentration-dependent effects of NO are not the only explanation, the interaction of NO with ROS may provide important clues as to mechanisms of specific in vivo pathways. Here we have examined the effects of NO-mediated signaling in relation to superoxide and hydrogen peroxide. We demonstrate that the primary consequence of O₂⁻ generation concomitant with NO production is not the toxicity associated with the formation of higher nitrogen oxides, but rather the resultant phenotypic cellular changes that occur because of limiting the bioavailability of NO and H₂O₂.

**EXPERIMENTAL PROCEDURES**

**Materials**—SOD (bovine erythrocytes), aminotriazole (ATZ), cytochrome c, catalase (CAT), hypoxanthine (HX), myoglobin (horse heart), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), diethylenetriaminopentaacetic acid (DTPA), nitro blue tetrazolium (NBT), and dimethylformamide (DMF) were purchased from Sigma-Aldrich. 2,3-Dimethoxy-1,4-naphthoquinone (ABTS), diethylenetriaminopentaacetic acid (DTPA), nitro blue tetrazolium (NBT), and dimethylformamide (DMF) were purchased from Sigma-Aldrich. 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) from Calbiochem. H₂O₂ was from Fisher Scientific. Other reagents were as follows: xanthine oxidase (XO; Roche Applied Science, Nutley, NJ), dihydrorhodamine 123 (DHR; Molecular Probes, Eugene, OR). Sper/NO (spermine nonoate) was a generous gift from Drs. J. Saavedra and J. A. Hrabie, NCI-FCRDC, Frederick, MD. The stock (prepared in 10 mM NaOH) concentration was determined immediately prior to use by measuring the absorbance at 250 nm (ε = 8,000 M⁻¹ cm⁻¹).

The NO/O₂⁻ Reaction—Superoxide was generated by XO-catalyzed degradation of HX (500 μM) in serum-free media. The rate of O₂⁻ production was determined by the rate of reduction of cytochrome c (550 nm, ε = 21,000 M⁻¹ cm⁻¹), as previously described. Under these conditions production of urate from xanthine was determined spectrophotometrically (305 nm; ε = 8030 M⁻¹ cm⁻¹) to be negligible. The calculated rate of NO release from 100 μM Sper/NO based on the decomposition rate (pH 7.4, 37 °C, t₁/₂ = 42 min) is 3.0 μM/min. The actual rate of 2.7 μM NO/min was verified by measuring oxyhemoglobin oxidation (582 nm, ε = 9,200 M⁻¹ cm⁻¹) in HX/serum-free media and by the decomposition of Sper/NO (250 nm (ε = 8,000 M⁻¹ cm⁻¹).

Measurement of steady-state concentrations of NO produced from Sper/NO during cell culture treatments was accomplished using a Seivers (Boulder, CO) NO gas analyzer. Aliquots of media (100 μl) from Sper/NO-treated cells were immediately injected into the reaction chamber containing 0.5 mM NaOH to stop NO donor decomposition and under anaerobic conditions (pumped with helium) to eliminate NO autooxidation. Steady-state molar NO concentrations were calculated from the absolute amounts of NO detected.

**SOD Activity Assay**—SOD activity was measured by the method of Spitz and Oberley (30). This assay is based upon the reduction of NBT to blue formazan by O₂⁻. One unit of SOD activity was defined as the amount required to yield 50% of sample maximum inhibition of NBT reduction by O₂⁻. The activity of the SOD we used was 178.6 units/μg indicating >90% functional SOD. H₂O₂ was measured by ferrous iron oxidation in the presence of xylene orange as previously described (31).

**Oxidation Assay**—The strong oxidants generated from the NO/O₂⁻ reaction were measured by formation of the fluorescent compound rhodamine (RH) via two-electron oxidation of DHR (32). Immediately prior to use, stock solutions of 50 mM DHR (10 mg) were prepared in DMF (0.6 ml) and diluted 1000-fold into cell culture plates. After various concentrations of XO and Sper/NO were added to the cultures, the reactions were allowed to proceed for 2 h at 37 °C (5% CO₂, 95%, air incubator). Fluorescence was measured at 570 nm following excitation at 500 nm. Measurements were made in the 6-well plates for total oxidation, or the media was removed and measured separately for extracellular oxidation, and PBS was replaced onto the cells (washed × 3) and measured to determine intracellular oxidation.

**Cell Culture**—MCF7 human breast carcinoma cells (ATCC) were grown to 85% confluency in 6-well culture plates with RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (Hyclone, Logan, UT). Prior to treatments, the cells were incubated in 2 ml of serum-free RPMI 1640 medium overnight. All treatments were conducted under these culture conditions.

**Co-culture experiments:** ANA-1 murine macrophages were activated with interferon-γ (10 ng/ml) for 16 h followed by LPS (10 ng/ml) for 4 h to induce iNOS expression. The cells were trypsinized, counted, and seeded on top of MCF-7 cells at various densities in serum-free media supplemented with either...
Superoxide Limits Nitric Oxide Signaling

L-arginine (1 mM) or aminoguanidine (5 mM). ANA-1 cells are HIF-1α and p53-null and therefore do not interfere with the measurement of these proteins in MCF7 cells. ANA-1 cells only loosely adheres to the culture flask and/or MCF-7 cells during the treatment period and are easily removed by gentle washing of the MCF7 cells with PBS at the termination of the experiment.

Human Umbilical Vein Endothelial Cells (HUVECs, Cambrex, East Rutherford, NJ) were cultured in EGM media supplemented with 5% fetal bovine serum, epidermal growth factor, bovine brain extract, cortisone, gentamycin, penicillin, and streptomycin and maintained at 37 °C in an atmosphere of 5% CO2 and room air. Prior to treatment, cells were trypsinized and plated at a density of 1 × 10^6 cells per 100-mm tissue culture dish and grown for 72 h. The cells were then washed with PBS and incubated in phenol red-free EGM media containing 5% fetal bovine serum, bovine brain extract, heparin, and penicillin.

Western Blot—Protein cell extracts were made by washing cells in cold PBS, scraping plates, centrifuging, and resuspending in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease inhibitor mixture; Calbiochem, San Diego, CA). Following 30 min of incubation on ice, the samples were centrifuged at 14,000 × g, and the supernatant protein concentration was determined by the bicinchoninic acid method (Pierce). Protein samples of equal amounts were subjected to PAGE on 10% Tris-glycine acrylamide gels (Novex-Invitrogen). Following transfer to polyvinylidene difluoride Immunolon P membranes (Millipore, Bedford, MA), samples were probed with rabbit polyclonal or mouse monoclonal antibodies (HIF-1α (Transduction Laboratories), ERK, p53 P-Ser-15, (Cell Signaling), HPRT (Santa Cruz Biotechnology). Bands were visualized with horseradish peroxidase-conjugated secondary antibodies (1:2,000–10,000; Sigma) and chemiluminescent substrate (Pierce). HPRT protein loading controls were run for each gel. Gels images were scanned using an AGFA DuoScan hiD scanner (Wilmington, MA) or Alpha Innotech Imaging system, and relevant bands were cropped to size using Photoshop 7.0 with no further manipulation. Densitometry was measured using AlphaEase® FC Stand Alone Software. Figures are representative of n ≥ 3 individual experiments.

Clonogenic Assay—Cell survival/proliferation was measured by clonogenic assay with plating efficiency ranging between 85 and 95% as previously described (33). Briefly, stock cultures of each cell type were plated (7 × 10^4 cells/100-cm2 dish) and incubated for 24 h prior to treatment. After treatments, cells were washed, trypsinized, counted, and plated in triplicate for macroscopic colony formation. Following a 10–14-day incubation period, colonies were fixed, stained, and counted. Colonies containing >50 cells were scored.

cGMP Measurement—cGMP was measured using the enzyme immunoassay (EIA) Biotrak system (Amersham Biosciences). Cells were grown in 96-well plates, serum-starved overnight and subjected to various treatment conditions. The cells were washed, lysed, and the supernatant transferred to the EIA plate for cGMP measurement.

RESULTS

Threshold NO Concentrations Regulate HIF-1α and p53—It has been demonstrated that NO causes p53 serine 15 phosphorylation and HIF-1α accumulation in a concentration-dependent manner (1, 4, 28). When increasing concentrations of the NO donor Sper/NO (25–100 μM) were added to MCF7 cells, both p53 P-Ser-15 and HIF-1α increased (Fig. 1). These changes were compared with the mean steady-state NO concentrations measured in the media of the Sper/NO-treated cells (Fig. 1). The concentration of NO necessary for HIF-1α stabilization and p53 P-Ser-15 correlated to the steady-state NO concentrations as previously reported (4).

HIF-1α and p53 Regulation by Other Radical/Oxidant Species—Because p53 P-Ser-15, HIF-1α, and ERK are regulated by distinct threshold concentrations of NO in MCF7 cells (4), we evaluated the effect of extracellular O2− and H2O2. Fig. 2 shows the effect of various RONS (NO, O2−, H2O2, ONOO−) on HIF-1α and p53 P-Ser-15 regulation in MCF7 cells. Treatment with the NO donor Sper/NO (100 μM, 2 h) showed both HIF-1α and p53 P-Ser-15 were stabilized as previously described (lane 2). However, in the presence of HX/XO, there was a decrease in NO-mediated HIF-1α accumulation but p53 P-Ser-15 was maintained (compare lane 6 and lane 2). These results suggest...
that ROS differentially modulate the effects of NO on these two proteins. To determine the direct effects of ROS on p53 and HIF-1α signaling, MCF7 cells were treated only with XO plus HX. p53 phosphorylation (P-Ser-15) was detected whereas HIF-1α was not (lane 3). Under these same conditions, with the addition of SOD, which converts O₂⁻ to H₂O₂, the results did not change, and only p53 P-Ser-15 was detected (lane 4). However, in the presence of catalase, which converts H₂O₂ to H₂O and O₂, neither HIF-1α nor p53 P-Ser-15 were detected (lane 5). These findings suggest that H₂O₂ but not O₂⁻ induces p53 P-Ser-15 stabilization but not HIF-1α.

Quantifying the Effect of H₂O₂ on p53 Phosphorylation in MCF7 Cells—Fig. 3A demonstrates the effect of increasing concentrations of bolus H₂O₂ on the activation of HIF-1α, p53 P-Ser-15, and pERK. Only p53 P-Ser-15 was detected after 2 and 4 h (not shown) at H₂O₂ concentrations ≥50 μM. This is consistent with the findings that only H₂O₂ from HX/XO mediates phosphorylation of p53. When repeated additions of 25 μM H₂O₂ were added every 30 min, p53 P-Ser-15 was detectable after 3 h in cells exposed to 2 or more additions of H₂O₂ (≥50 μM accumulative H₂O₂) (data not shown). This indicated that the duration of exposure to H₂O₂ is equally as important as its concentration.

To better define the temporal dependence of H₂O₂-mediated p53 phosphorylation, cells were treated with H₂O₂ for different time intervals. The cells in Fig. 3B were treated with 100 μM H₂O₂. The media was then removed at the indicated time points and replaced with fresh media. All cells were incubated and harvested 8 h after treatment. These data indicate that ≤15 min of H₂O₂ exposure was required to elicit phosphorylation of p53, after which they are committed, and p53 P-Ser-15 was detected for ≥8 h. Phosphorylation of p53 Ser-15 occurred as little as 15 min after H₂O₂ exposure (Fig. 3C). Thus, H₂O₂ initiates a sustained signal cascade that manifests itself rapidly after treatment.

H₂O₂ Metabolism and p53 Signaling in MCF7 Cells—We previously reported that the duration of exposure time to NO is critical in the activation of p53 P-Ser-15 (4). It was important to determine if this was true for H₂O₂. To estimate the exposure time for cells upon bolus addition of H₂O₂ we first determined the rate of H₂O₂ metabolism by MCF7 cells in culture. MCF7 cells were exposed to bolus addition of H₂O₂ and the loss of H₂O₂ was quantified. Fig. 4, A and B demonstrates that when 100 μM bolus of H₂O₂ was added to MCF7 cells in culture, it was metabolized in a first-order manner with a rate of $k_{obs} = -0.0649 \text{ min}^{-1}$, $-1.73 \times 10^{-7} \text{ min}^{-1} \text{ cell}^{-1}$. Fig. 4C shows that this rate was proportional to the two-dimensional density of the MCF7 cell monolayer. When aminotriazole, a catalase inhibitor, was added, the rate of H₂O₂ metabolism decreased (Fig. 4C) similar to what has been reported previously for other cell types (34).

Unlike NO, it appears that cell membranes pose important limiting barriers for the partitioning and diffusion of H₂O₂ (35–37). To test this, MCF7 cells were harvested and placed in suspension at various concentrations to increase their density. Fig. 3D demonstrates that even in the presence of $1 \times 10^7$ MCF7 cells/ml, p53 P-Ser-15 was detected after 2 h in the presence of 100 μM H₂O₂. The half-life of NO at this cell density is calculated to be <0.5 s (37), far too short to exert an effect on p53 phosphorylation. This demonstrates that the rate of H₂O₂ metabolism is slow relative to the rate of NO metabolism and therefore the exposure time of cellular targets to H₂O₂ is greater than that for NO.
It has been shown previously that H$_2$O$_2$ is primarily metabolized in cells by catalase and glutathione peroxidase (34). Pharmacologic catalase inhibition should therefore further extend the half-life (exposure time) of H$_2$O$_2$ and increase its biological effects (Fig. 4). When MCF7 cells were incubated for 3 h with increasing H$_2$O$_2$ concentrations, a dose-dependent increase in p53 P-Ser-15 was observed (Figs. 3 and 5). In the presence of aminotriazole, a potent catalase inhibitor, the minimum dose of H$_2$O$_2$ necessary to induce p53 phosphorylation was decreased and the response at higher concentrations was markedly more robust (Fig. 5).

Because both NO and H$_2$O$_2$ independently induce p53 phosphorylation, we expected the result to be additive when both molecules were present simultaneously. Remarkably, concurrent NO and H$_2$O$_2$ exposure demonstrated the opposite effect on p53 phosphorylation (Fig. 5). When increasing concentrations of H$_2$O$_2$ were incubated with NO (Sper/NO 50 μM) the response of p53 was suppressed in MCF7 cells. NO did not affect the rate of H$_2$O$_2$ metabolism (data not shown). These surprising results indicate that NO and H$_2$O$_2$ antagonize each other’s activity to induce p53 phosphorylation.

Oxidants Formed from the Reaction of NO with O$_2$—The chemistry of NO and O$_2$ dictates that the oxidants formed...
Superoxide Limits Nitric Oxide Signaling

The effect of NO concentration versus oxidant formation on the post-translational regulation of p53 and HIF-1α. MCF7 cells were grown in 6-well plates and serum-starved overnight. They were then loaded with DHR 50 μM and treated for 2 h with: A, with increasing concentrations of Sper/NO (1–100 μM); B, increasing concentrations of Sper/NO (1–100 μM) + O2− (~1.35 μM O2−/min); and C, increasing concentrations of Sper/NO (1–100 μM) + O2− (~1.35 μM O2−/min) + SOD 20 μM (2.3 × 105 units). At the end of the treatments, steady-state NO concentrations were measured, intracellular and extracellular rhodamine formation was measured and cell extracts were isolated for Western blot analysis. HIF-1α and p53 P-Ser-15 were measured by Western blot (n = 3).

![Figure 7](image)

from this reaction are at a maximal yield when the fluxes of both radicals are equal. When the flux of either radical is varied with respect to the other, a characteristic bell-shaped oxidation profile exists (38, 39). Fig. 6 confirms the bell-shaped oxidation curve that was achieved in the presence of MCF7 cells when NO and O2− were produced concomitantly. At a constant O2− flux (~1.35 μM O2−/min), increasing the rate of NO production first increased the level of DHR oxidation and then decreased DHR oxidation when NO was in excess. The curve in Fig. 6 represents the sum of both intra- and extracellular DHR oxidation. After washing to remove extracellular DHR, the shape of the intracellular oxidation curve remained the same, and only its magnitude decreased (data not shown), similar to that previously reported (40, 41). When SOD was present, the shape of the curve flattened as O2− was converted to H2O2 before it could react with NO. Because oxidant formation could be accurately determined from the co-generation of NO and O2−, the oxidant profile was correlated with the post-translational regulation of p53 and HIF-1α under these conditions.

Extracellular O2− Generation Restricts NO Signaling—We treated MCF7 cells with increasing concentrations of the NO donor Sper/NO (1–100 μM) for 2 h. Under these conditions, we were able to simultaneously measure both DHR oxidation and O2−, it was found that HIF-1α required constant NO exposure, whereas p53 P-Ser-15 stabilization, like with H2O2, was initiated by NO but remained elevated long after exposure (Fig. S1, supplemental data).

Intracellular O2− Generation Restricts NO Signaling—Having established that extracellularly generated O2− can have substantial effects on the magnitude of post-translational regulation of HIF-1α and p53 by NO, the effects of intracellularly produced O2− were examined. Exposing DHR-treated MCF7 cells to NO and DMNQ or menadione, both known generators of intracellular O2− (34), resulted in measurable increases in DHR oxidation (Fig. S2, supplemental data). Fig. 8A shows the regulation of HIF-1α and p53 P-Ser-15 in the presence of NO alone or NO + menadione. This figure demonstrates that intracellularly generated O2− shifts the threshold for Sper/NO-mediated HIF-1α and p53 Ser-15 regulation toward higher concentrations, analogous to the effect of HX/XO. Thus, higher NO production is required to activate HIF-1α and p53 in the presence of intracellular O2− production. As a control, CoCl2, a potent and NO-independent activator of HIF-1α, was used. The increase in HIF-1α by CoCl2 treatment was unaffected by menadione. Taken together, these findings imply that decreases in HIF-1α levels in the presence of O2− generated...
Superoxide Limits Nitric Oxide Signaling

from menadione result from NO scavenging rather than $\text{O}_2^-$ directly destabilizing HIF-1$\alpha$. These conditions necessitated an increased rate of NO production to induce HIF-1$\alpha$ stabilization in the presence of $\text{O}_2^-$.

NO-mediated Guanylyl Cyclase Activation Is Limited by $\text{O}_2^-$ and Enhanced by SOD—Because sGC is one of the most important and sensitive biological targets for NO, it follows that $\text{O}_2^-$ should change the threshold concentration for activation by NO. Intracellular cGMP levels in MCF7 cells were determined after 30 min of exposure to NO. We found that maximal sGC activation required ≥5 μM Sper/NO (Fig. 8B). However, the presence of extracellular (HX/XO) or intracellular (DMNQ) $\text{O}_2^-$ resulted in higher concentrations of Sper/NO necessary to activate sGS. In the presence of XO, 10 times higher Sper/NO concentration was required to achieve the same level of sGC activation as without. When SOD was present during NO and extracellular $\text{O}_2^-$ production, the sensitivity of sGC to NO was enhanced. The presence of SOD alone increased the sensitivity of sGC to NO indicating that basally produced $\text{O}_2^-$ is modulating NO-mediated sGC activation. Similarly, when $\text{O}_2^-$ was generated intracellularly, the sensitivity of sGC to NO was lost. Unlike extracellularly produced $\text{O}_2^-$, when it was produced intracellularly, the addition of SOD had a minimal effect on restoring the sensitivity of sGC to NO (<10% of maximal cGMP produced). Because DMNQ-generated $\text{O}_2^-$ is unaffected by the addition of SOD, this confirms that $\text{O}_2^-$ is predominantly formed intracellularly and reacts with NO.

$\text{O}_2^-$ Antagonizes Signaling Mediated by Macrophage-derived NO—NO-producing cells are common to many inflammatory and malignant conditions. Various studies have used activated macrophages as a biological source of NO production (4, 42), and they were used in this study to examine the effects of $\text{O}_2^-$ generation on NO signaling events. When cytokine (IFN$\gamma$/LPS)-activated NO-producing ANA-1 cells were co-cultured with MCF7 cells at 1:4 and 1:8 ratios, both HIF-1$\alpha$ and p53 P-Ser-15 were detected in the ANA-1 cells supplemented with 1 mM l-arginine were seeded into MCF7 cell cultures at the indicated ratios. XO was added (HX 500 μM (~1.35 μM $\text{O}_2^-$/min) as indicated. Lanes: 1, MCF7 alone; 2, ANA-1 alone; 3, activated ANA-1 alone; 4, MCF7/ANA-1 1:4 + HX; 5, MCF7/ANA-1 1:4 + HX/XO; 6, MCF7/ANA-1 1:8 + HX; 7, MCF7/ANA-1 1:8 + HX/XO. Cells were treated for 3 h, and proteins were extracted for Western blot analysis ($n = 3$).

$\text{O}_2^-$ Antagonizes NO Signaling in Endothelial Cells—The effect of NO/$\text{O}_2^-$ on p53 P-Ser-15 and HIF-1$\alpha$ was also examined in endothelial cells. Fig. 10 illustrates the dramatic influence $\text{O}_2^-$ had on NO-induced HIF-1$\alpha$, and p53 in HUVEC cells. In the presence of NO alone, HIF-1$\alpha$ and p53 are relatively sensitive to its effects. Yet during concomitant low fluxes of $\text{O}_2^-$, the rate of NO production necessary to elicit the same signal transduction responses was greatly increased. These results in HUVECs suggest a general mechanism in response to NO/$\text{O}_2^-$ chemistry. In endothelial cells, NO at high concentration reduces pERK through increasing its corresponding phosphatase MKP-1 (5). The concentration of Sper/NO that activates p53 also decreased pERK. However, in the presence of $\text{O}_2^-$, pERK was maintained, suggesting that NO levels are critical in...
regulating pro- and antigrowth mechanisms in endothelial cells.

**NO Protects Cells from O$_2^-$/H$_2$O$_2$-induced Cytotoxicity**—Because NO and ROS can generate potentially toxic species, clonogenic assays were performed to determine the potential long-term cytotoxic/cytostatic effects of our various treatment paradigms. Fig. 11 summarizes our results. NO alone was non-toxic at 100 μM Sper/NO consistent with other reports (43, 44). The cytotoxicity of H$_2$O$_2$ alone was completely prevented by the addition of NO. Low nanomolar concentrations of intra- or extracellular O$_2^-$ alone, or in combination with NO at varying ratios, were also non-toxic. These results demonstrate that physiologically relevant concentrations of NO production are protective rather than toxic. Even the formation of strong oxidants, as measured by DHR, from the reaction of NO with O$_2^-$ had no effect on cell survival. Furthermore, the formation of powerful nitrating intermediates from the reaction of NO with O$_2^-$ is purported to have major biological and pathological consequences (45). We measured the formation of 3-nitrotyrosine in all of the samples in Fig. 7. We were unable to detect 3-nitrotyrosine by Western blot analysis from total cell lysates in any of the samples (data not shown) consistent with previous reports (12). These findings reconfirm the important antioxidant rather than pro-oxidant properties of NO (43).

**DISCUSSION**

Herein we report an alternative physiologic function for the reaction between NO and O$_2^-$ with respect to signal transduction. Much debate and controversy has arisen regarding the physiologic and pathologic consequences of the strong nitrative and oxidative intermediates formed from this reaction. Some previous work has emphasized the potential toxic and deleterious nature of the isolated chemical species formed from the NO/O$_2^-$ reaction. Our data instead demonstrate that this reaction is not a significant source of toxic species, but rather a mechanism to regulate steady-state NO levels and signal transduction events.

NO-regulated proteins have different sensitivities to steady-state concentrations of NO. Therefore, the rate of O$_2^-$ production is critical in this paradigm because O$_2^-$ concentrations determine the steady-state concentration of NO. A greater rate of NO production is necessary in the presence of O$_2^-$ to achieve the same cellular response as in the absence of O$_2^-$.

Superoxide generation will therefore alter the signaling response to NO by directly altering its steady-state concentration. Both the chemical data and cellular responses suggest that quantitatively the most biologically significant consequence of this reaction is the change in steady-state NO concentration because of its reaction with O$_2^-$. The elegance of this reaction lies in its simplicity and the importance, therefore, is not a function of the resultant chemistry from the formation of higher nitrogen oxides, but rather the alterations in cellular phenotype because of the attenuation in bioactive NO levels.

One of the earliest examples of this paradigm was the observation that O$_2^-$ decreased cGMP levels and inhibited NO-mediated vascular smooth muscle relaxation (46). This eventually led to the identity of NO as endothelial-derived relaxation factor. Recently Kohl et al. (47) have demonstrated HIF-1α regulation by NO is determined by intracellularly generated O$_2^-$. Conversely, just as O$_2^-$ abates NO signaling, NO had been shown to influence O$_2^-$-mediated signaling as well as. For example: activation of Erg1, ICAM-1, and MCP-1 by O$_2^-$ in endothelial cells is abated by NO (48, 49). In summary, NO and O$_2^-$ work in concert, each co-regulating the concentration of the other.

High concentrations of NO have been shown to inhibit tumor (50, 51) and endothelial cell growth (5) through increases in p53 phosphorylation and MKP-1 activation. Low NO concentrations tend to favor pro-growth pathways that protect against apoptosis, whereas at high relative steady-state NO levels, p53 will be phosphorylated resulting in cell cycle arrest, and potentially apoptosis. Intermediate levels of NO will cause ERK phosphorylation and HIF-1α accumulation, which are associated with a mitogenic, angiogenic, growth phenotype. Lower nanomolar steady-state NO levels will activate sGC with the subsequent formation of cGMP. Therefore, high NO levels are generally inhibitory whereas lower levels are stimulatory.
Superoxide Limits Nitric Oxide Signaling

Despite the formation of RNOS, the primary effect of O$_2^\cdot$ on signaling induced by higher levels of NO is to abate anti-proliferative mechanisms and to enhance pro-growth systems.

An important mechanism described here is that not all proteins have the same response to a given redox stimulus. Susceptible proteins respond to NO and/or oxidative stress either transiently or constitutively. For example, the regulation of HIF-1$\alpha$ or cGMP by NO parallels the dose and duration of NO exposure. When NO is removed or falls below the concentration threshold necessary for activation, HIF-1$\alpha$ levels decline. However, p53 phosphorylation by NO (>300 nM for over 2 h) or H$_2$O$_2$ (20–40 $\mu$M for <15 min) will remain elevated long after exposure. This becomes important when considering the temporal effects of O$_2^\cdot$ exposure on various NO-regulated proteins. P53 P-Ser-15, once activated by NO, will remain elevated even during subsequent O$_2^\cdot$ exposure. However, HIF-1$\alpha$, which requires constant NO exposure, will therefore be sensitive to the temporal and NO-scavenging effects of O$_2^\cdot$. This suggests that certain cellular proteins will be more affected than others by acute changes in the local redox environment where NO is suspected to be a major driving force. Redox signaling (p53) induced by stress levels of NO or H$_2$O$_2$ are persistent, but pro-growth and anti-apoptotic signals (cGMP, HIF-1$\alpha$) are more reversible. This suggests that the timing of NO and O$_2^\cdot$ generation may be equally important for understanding the mechanisms of redox-mediated signaling in relation to tissue growth and injury.

Numerous studies have shown that NO has powerful antioxidant properties serving to suppress chemical and biological mechanisms associated with oxidative stress. Of all the NO/ROS treatment paradigms explored in this study, H$_2$O$_2$ exposure was most toxic, resulting in only $\approx$10% surviving fraction. Interestingly, although both NO and H$_2$O$_2$ induce p53 P-Ser-15, only H$_2$O$_2$ resulted in cell death, indicating that additional signaling targets determine the cellular consequences of p53 activation. Conversely, O$_2^\cdot$ or NO alone or in combination at various flux ratios was not toxic despite the formation of strong oxidants. Furthermore, the toxicity of H$_2$O$_2$ was completely prevented if NO was present during treatment and the induction of p53 P-Ser-15 was abolished. These results go against the current dogma regarding free radicals and disease by highlighting the antioxidant and protective properties of NO. The presence of nitrotyrosine, as evidence for the reaction of NO with O$_2^\cdot$, has been involved in the etiology of many disease states, and its formation is often considered to be the precipitating or causative event (45, 52, 53). We were unable to detect 3-nitrotyrosine after co-generation of various low flux ratios of NO and O$_2^\cdot$; nor did these treatments result in decreases in cell survival or viability. Quantitatively, the effects of 3-nitrotyrosine formed from this reaction are negligible compared with the bio-regulatory aspects.

It is important to note that whereas NO affords protection against acute exposure to toxic levels of O$_2^\cdot$/H$_2$O$_2$, the long-term ramifications of these interactions may be of considerable consequence. Deviations from normal NO signaling in response to O$_2^\cdot$ production can dramatically alter the long-term outcome. When p53-inducing levels of NO are reduced to concentrations only capable of activating cGC and HIF-1$\alpha$, the cell is essentially rendered p53-null and it is transformed from an inhibitory to a pro-growth phenotype. It should be emphasized that it is not the toxicity of the intermediates from these reactions that are important in disease progression but the potential long-term changes in cellular phenotypes.

There is much evidence demonstrating the roles of NO and O$_2^\cdot$ in various mechanisms of carcinogenesis and tumor biology. Macrophage-derived NO is essential for the suppression of some tumors (54). Recent studies have shown that there is a higher incidence of lymphomas in p53/NOS-2 double knock-out mice (55). Yet there was a significant delay in the onset of tumors in p53$^{-/-}$ mice that were fed TEMPO, an SOD mimetic (56). These findings suggest a link between p53 regulation and redox status in vivo such that higher levels of NO may be important in suppressing tumorigenesis. Abatement of ROS may also be important. Because p53 is elevated by NO (breast, TK6, Ref. 27, etc.), it stands to reason that limiting oxidative stress in part increases NO. Some tumors have been associated with increased oxidative stress, which may serve to abate NO-mediated killing. It has been shown that NOS-2 transfection limits tumor growth and is directly related to NO production (20, 24, 27). Thus, the antitumor activity of NO correlates to high NO and appears to be opposed by oxidative stress.

Angiogenesis is also necessary for tumor progression. Soluble guanylyl cyclase is activated by low nanomolar levels of NO and appears to play a central role in this process. In the presence of O$_2^\cdot$, generated intracellularly or extracellularly, the concentration of NO had to be substantially increased to achieve the same level of cGMP formation as in the absence of O$_2^\cdot$ (17). Low levels of NO can also stimulate proliferation of endothelial cells and reduce the transcription of antiangiogenic factors such as TSP-1. Although HIF-1$\alpha$ and subsequent VEGF production are increased by NO in endothelial cells, higher concentrations of NO antagonize this by increasing p53 phosphorylation and MKP-1 as well (5, 51). Again we find that O$_2^\cdot$ generation inverts the NO-linked phenotype. These are effects that were reversed by the addition of SOD. This demonstrates the profound effect low levels of O$_2^\cdot$ can have on NO signaling. These data also illustrate the pathological consequences of aberrant O$_2^\cdot$ generation or the absence of SOD. In the face of excess O$_2^\cdot$, the induction of cGMP-mediated signal transduction pathways by moderate levels of NO may be abolished.

In conclusion, these results demonstrate an alternate yet fundamental role of O$_2^\cdot$ in regulating NO pathophysiology. The primary consequence of O$_2^\cdot$ production may simply be the result of reducing the bioavailability of NO. Because most NO-regulated proteins appear to be exquisitely sensitive to concentration effects, alterations in NO levels as a result of excess O$_2^\cdot$ may have profound phenotypic effects. The generation of NO and O$_2^\cdot$ appear to be mostly non-toxic, and in all cases tested NO was protective. These results also demonstrate that not only will the actual radical fluxes effect cellular signaling, but indirectly, so will the presence of antioxidant enzymes (SOD, catalase). These results can be extended to many physiologic and pathologic circumstances, and they become especially important when examining conditions attributed to excess radical production or alterations in antioxidant defense systems.
