Superoxide Production and Reactive Oxygen Species Signaling by Endothelial Nitric-oxide Synthase*

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Weihan Wang, Shuibang Wang, Liang Yan‡, Patricia Madara, Ana Del Pilar Cintron, Robert A. Wesley, and Robert L. Danner§

From the Critical Care Medicine Department, Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland 20892

Reactive oxygen species can function as intracellular messengers, but linking these signaling events with specific enzymes has been difficult. Purified endothelial nitric-oxide synthase (eNOS) can generate superoxide (O2•−) under special conditions but is only known to participate in cell signaling through NO. Here we show that eNOS regulates tumor necrosis factor α (TNFα) through a mechanism dependent on the production of O2•− and completely independent of NO. Expression of eNOS in transfected U937 cells increased phosphor 12-myristate 13-acetate-induced TNFα promoter activity and TNFα production. Nω-Methyl-L-arginine, an inhibitor of eNOS that blocks NO production but not its NADPH oxidase activity, did not prevent TNFα up-regulation. Likewise, Glu361eNOS, a competent NADPH oxidase that lacks NOS activity, retained the ability to increase TNFα. Similar to the effect of eNOS, a O2•− donor dose-dependently increased TNFα production in differentiated U937 cells. In contrast, cotransfection of superoxide dismutase with eNOS prevented TNFα up-regulation, as did partial deletion of the eNOS NADPH binding site, a mutation associated with loss of O2•− production. Thus, eNOS may straddle a bifurcating pathway that can lead to the formation of either NO or O2•− interrelated but often opposing free radical messengers. This arrangement has possible implications for atherosclerosis and septic shock where endothelial dysfunction results from imbalances in NO and O2•− production.

Endothelial nitric-oxide synthase (eNOS)1 is a calcium-dependent NADPH oxidase that generates NO from oxygen and L-arginine (1, 2). It has a C-terminal reductase domain that binds NADPH, FAD, and FMN, and a N-terminal oxygenase domain that binds a heme moiety, tetrahydrobiopterin (BH4), and l-arginine (3). NO produced by eNOS regulates vascular tone through a cGMP-dependent signaling pathway (4). In addition, NO also has cGMP-independent effects within the vasculature, such as inhibition of leukocyte adhesion, that relies on its ability to inactivate or antagonize O2•− (5, 6). Notably, in cell free systems, purified eNOS can be shown in the absence of BH4 to generate small quantities of O2•− rather than NO (7, 8). However, cell regulation directly attributable to eNOS production of O2•− has not been previously demonstrated.

Superoxide (O2•−) and other reactive oxygen species (ROS), conventionally viewed as cytotoxins, have recently been recognized as important signal transduction intermediates that regulate gene expression, cell differentiation, immune activation, and apoptosis (9–13). In the vasculature, increased O2•− production disproportionate to NO synthesis has been associated with endothelial dysfunction, an early pathogenic event in atherosclerosis (14–17). Further, elevated O2•− production in the presence of NO has been linked to endothelial injury and organ damage in septic shock (18), possibly through the formation of peroxynitrite (ONOO−), a cytotoxic metabolite (19–22). However, the precise enzymatic origins of ROS in either signaling events or pathologic conditions have remained obscure (23, 24). ROS have many potential sources within cells including mitochondria, xanthine oxidase, cyclooxygenases, and NADPH oxides, making it difficult to associate a specific enzyme with a corresponding ROS-related event (23, 24).

We have previously described the expression of functional human eNOS in U937 cells using a pCEP4 vector (25). Human monoblastoid U937 cells lack NOS expression in either their naïve or phosphor 12-myristate 13-acetate (PMA) differentiated states (26). At resting levels of intracellular calcium, eNOS expression in transfected U937 cells does not result in NO release, but NO is rapidly produced upon exposure to calcium ionophore (25). The absence of soluble guanylate cyclase (26), a pivotal target of NO-based signaling, and impaired synthesis of BH4 (27), a cofactor whose deficiency is associated with O2•− production by eNOS in cell-free systems (7, 8), suggested that these cells might be useful for exploring NO-independent signaling by eNOS.

EXPERIMENTAL PROCEDURES

Reporter Assay—A firefly luciferase reporter vector pGL2 (Promega) containing the full-length human tumor necrosis factor α (TNFα) promoter (pTNF-G2) was used to assay for TNFα promoter activity. pTNF-G2 (20 μg) and a vector (5 μg) for expression of secreted placent al alkaline phosphatase (to control for transfection efficiency) were cotransfected into U937 cells with either the eNOS pCEP4 construct (20 μg) or the empty pCEP4 vector (20 μg) using electroporation (240 V, 960 microfarad). Then cells were differentiated with 100 nM PMA for 48 h, and luciferase and secreted placental alkaline phosphatase activity were measured (Promega and Tropix Inc).

Expression Vectors—The human eNOS expression vector was constructed by using the eukaryotic expression vector pCEP4 (Invitrogen) (25). A Glu361→Gln361 mutant eNOS cDNA was ligated into pCEP4 at HindIII/NotI sites to create Gln361eNOS. Human Cu/Zn superoxide dismutase (SOD) cDNA (American Type Culture Collection) was ligated...

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‡ Present address: Dept. of Pathophysiology, Medical School of Jinan University, Guangzhou 510632, People’s Republic of China.
§ To whom correspondence should be addressed: Critical Care Medicine Dept., NIH, Bldg. 10, Rm. 7D43, 10 Center Dr., MSC 1662, Bethesda, MD 20892-1662. Tel.: 301-496-9320; Fax: 301-402-1213; E-mail: rdanner@nih.gov.

1 The abbreviations used are: eNOS, endothelial nitric-oxide synthase; BH4, tetrahydrobiopterin; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; TNFα, tumor necrosis factor α; SOD, superoxide dismutase; L-NMA, Nω-methyl-l-arginine.
into pCEP4 at HindIII/BamHI sites to generate the SOD expression vector. eNOS NADPH binding site deletion mutants, d(NADPH) eNOS and d(NADPH) Gln361eNOS, were constructed by digesting eNOS and Gln361eNOS with XhoI, thereby removing a 717-base pair fragment from eNOS containing the sequence for its NADPH-adenine binding site. All expression vectors were partially sequenced to confirm the correct sequence and orientation.

Transfection—U937 cells (American Type Culture Collection) were maintained in RPMI 1640 complete medium containing HEPES (25 mM), 10% fetal calf serum, L-glutamine (2 mM) and antibiotics. Empty pCEP4 (control vector) or expression vectors containing human eNOS, Gln361eNOS, CuZnSOD, d(NADPH)eNOS, or d(NADPH)Gln361eNOS were transfected into cells by electroporation and then selected with hygromycin B (275 units/ml; Calbiochem), as described previously (25).

TNFα Production—After selection, transfectants (1 × 10^6 cells each) were suspended in 100 ml of RPMI 1640 complete medium and 100 nM PMA for 48 h. Adhered cells were removed by incubation with 1 mM EDTA in Hanks’ balanced salt solution without Ca^2+ and Mg^2+ for 30 min at 37 °C. Cells were then washed with Hanks’ balanced salt solution without Ca^2+ and Mg^2+, resuspended in RPMI 1640 complete medium, counted, tested for viability, and plated into 24-well plates at 5 × 10^5 cells/ml for 22 h. In one experiment, untransfected U937 cells were differentiated with PMA, as described above, and then incubated for 22 h with increasing doses of phenazine methosulfate, a O2 donor (28), for 0.5 h. Supernatants were assayed for TNFα using an enzyme-linked immunosorbent assay (R & D Systems).

NOS Activity and Aconitase Assay—NOS activity was determined by measuring the conversion of [14C]L-arginine to [14C]L-citrulline in total cell lysates from PMA-differentiated U937 cells, as reported previously (25). For the aconitase assay, differentiated cells incubated in RPMI complete medium for 22 h were removed by scraper, washed with ice-cold Hanks’ balanced salt solution without Ca^2+ and Mg^2+, and disrupted using a microtip sonicator. Lysate supernatants were assayed for aconitase activity as described elsewhere (28).

Statistics—Data are shown as the means ± S.E., and differences were considered significant where p < 0.05. Paired comparisons were performed using t tests. Dose response effects were analyzed using a Sen-Testi estimate of slope followed by a one-sample t test. Three-way comparisons were made using t tests followed by Holm’s multiple comparisons adjustment. Two-way analysis of variance followed by Fisher’s least significant difference test was used for higher order comparisons.

RESULTS AND DISCUSSION

Effect of eNOS on TNFα—Differentiation of U937 cells with PMA (100 nM) induces development of monocyte characteristics and TNFα production (29). To determine the effect of eNOS expression on TNFα promoter activity, we used a luciferase reporter system in PMA-differentiated U937 cells. As shown in Fig. 1A, eNOS expression increased TNFα promoter activity as compared with control vector transfectants (p = 0.0004). Next we examined whether this increase in TNFα promoter activity resulted in increased TNFα protein production (Fig. 1B). PMA-differentiated, eNOS-transfected U937 cells produced more TNFα compared with control vector-transfected cells (p = 0.01). However, N2-methyl-L-arginine (l-NMA), an inhibitor that blocks the NOS activity of eNOS but not its NADPH oxidase activity (30), did not prevent eNOS enhancement of TNFα production. These findings suggested that eNOS produced a signaling molecule other than NO that up-regulated TNFα production and raised the possibility that this alternative messenger was related to the NADPH oxidase activity of eNOS.

Effect of Gln361eNOS on TNFα—To further eliminate the possibility that NO was responsible for TNFα up-regulation by eNOS, we tested the mutant Gln361eNOS, which contains a single amino acid substitution from Gln to Gln at position 361 in the L-arginine binding site of wild type eNOS. This mutation abolishes the ability of eNOS to produce NO, but NADPH oxidase activity remains intact (31). Expression of Gln361eNOS (Fig. 1C) up-regulated TNFα production compared with control vector cells (p = 0.01) and had an effect similar to that of wild type eNOS (p = 0.5). Fig. 1D shows that wild type eNOS and Gln361eNOS were both expressed by their respective transfectants.

Effect of an O2 Donor or SOD Overexpression on TNFα Up-regulation—Next, phenazine methosulfate, a O2 donor (28), was shown to increase TNFα production in a dose-dependent manner (Fig. 2A; p = 0.001). This result was consistent with the possibility that ROS generated by eNOS could play a role in TNFα production. To further test whether O2 released by eNOS might be responsible for TNFα up-regulation, Cu/Zn SOD was cotransfected into U937 cells. In PMA-differentiated transfectants (Fig. 2B), SOD expression was found to totally abolish TNFα up-regulation by eNOS (p = 0.007). eNOS expression was not affected by the coexpression of Cu/Zn SOD (Fig. 2C).
These data suggest that eNOS-induced TNFα production required the production of O2− or a closely related metabolite.

**Effect of Deleting the NADPH Binding Site of eNOS**—To investigate the role of NADPH oxidase activity in TNFα up-regulation, the adenine binding site for NADPH was deleted from eNOS. Two NADPH binding site deletion mutants, d(NADPH)eNOS and d(NADPH/Gln361)eNOS, were constructed, one from wild type eNOS and the other from Gln361eNOS. Again we found that wild type eNOS expression (Fig. 3A) increased TNFα production compared with control vector (p = 0.002). However, neither d(NADPH)eNOS nor d(NADPH/Gln361)eNOS expression significantly altered TNFα production (p = 0.4). This result indicates that the NADPH oxidase activity of eNOS, which requires an intact NADPH recognition site, was necessary for TNFα up-regulation in PMA-differentiated U937 cells.

**NOS Activity and O2− Production**—Finally, NOS activity and O2− production were measured by l-arginine to l-citrulline conversion (25) and by acenitase assay (28, 32), respectively, to determine which enzymatic function corresponded to the ability of transfectants to up-regulate TNFα. The presence of NOS activity in cells transfected with wild type human eNOS or with one of the eNOS mutants did not correlate with the capacity to up-regulate TNFα (Fig. 4A). Only wild type eNOS transfectants were found to have NOS activity compared with control cells (p = 0.001). Although the Gln361eNOS mutant also up-regulated TNFα (Fig. 1C), it was completely devoid of NOS activity (Fig. 4A; compared with control p = 0.3). Next, suppression of acenitase activity was used as a measure of intracellular O2− production (28, 32). Aconitase activity in both wild type eNOS transfectants and Gln361eNOS mutants was lower than that of control vector transfectants (p < 0.0003 for both), indicating the presence of increased O2− production (Fig. 4B). In contrast, both d(NADPH)eNOS and d(NADPH/Gln361)eNOS transfected U937 cells, which do not produce increased amounts of TNFα (Fig. 3A), had acenitase activity similar to that measured in control vector transfectants (Fig. 4B; p = 0.1, for both). This shows, as expected, that the NADPH binding site mutants of eNOS did not produce increased amounts of O2−. Notably, l-NMA, a NOS inhibitor, did not change the acenitase activity pattern of eNOS transfectants (p = 0.8), demonstrating that decreases in acenitase activity in eNOS transfectants relative to control vector were not related to NO production (Fig. 4C) (19). Further, Fig. 4D shows that eNOS and its respective mutants were expressed in the PMA-differentiated U937 cells used in these experiments. Thus, the ability of various eNOS transfectants to up-regulate TNFα corresponded to their capacity to generate O2− but not NO.

Many cell types including endothelial cells, fibroblasts, hepatocytes, and vascular smooth muscle cells have been shown to utilize ROS as second messengers (9–12, 23, 24). These ROS-dependent signal transduction pathways have been shown to regulate important cellular functions such as growth, differentiation, gene expression, and apoptosis. However, even as the signaling mechanisms distal to ROS release are becoming increasingly well defined, identification of the precise enzymatic sources for ROS involved in specific signal transduction events has been relatively elusive (23, 24). The results presented here demonstrate that eNOS may be a source of O2− within cells and that eNOS-generated ROS can participate in the modulation of inflammatory responses.

eNOS has close homology with cytochrome P-450 reductases (1, 2) and can generate small amounts of O2− in cell-free systems deficient in l-arginine and BH4 (7, 8). In intact cells and in vivo, the availability of substrate and cofactors might be expected to keep this alternative electron shunting pathway inactive. However, several investigations (14–17) using human endothelial cell cultures, canine coronary arteries, or aortic rings from rats and rabbits are consistent with the possibility that eNOS might be capable of releasing oxygen intermediates under certain pathological conditions. Notably, the infusion of BH4 into patients with hypercholesterolemia was shown to restore endothelial-dependent vascular responses (15), supporting the con-
formation might occur to H2O2 and effectively blocked eNOS-may contribute to this response. Manna and other ROS molecules derived from it, in particular produces peroxynitrite (ONOO−). Notably, vessel shear stress has been shown to increase endothelial cell expression of both Cu/Zn SOD and eNOS (34). Co-regulation of these genes might serve to reduce the prooxidant potential of eNOS and to thereby maximally suppress oxidant-triggered apoptosis (34).

In the current investigation, we have shown that eNOS can generate small amounts of O2•− in intact cells. This eNOS-derived O2•− participated in cell signaling events that regulated the production of TNFα, a pivotal mediator of inflammation. Depending on the cell-type and triggering event investigated, ROS have been shown to transduce signals through the activation of tyrosine kinases, mitogen-activated protein kinases, and e-Jun N-terminal kinases and ultimately to alter promoter activity by increasing the DNA binding of transcription factors such as NFκB and AP-1 (9–13). In recent experiments, we have found in U937 cells that eNOS-based O2•− signaling activates p44/42 mitogen-activated protein kinase (35). Therefore, eNOS may occupy a signal bifurcation point controlling the production of two interrelated but distinct free radical messengers. Determining whether this arrangement exists in endothelium and, if so, how it is regulated might lead to an understanding of its possible benefits, given the potential danger it poses for the formation of ONOO−.

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