Evidence for peroxynitrite as a signaling molecule in flow-dependent activation of c-Jun NH2-terminal kinase

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Go, Young-Mi, Rakesh P. Patel, Matthew C. Maland, Heonyong Park, Joseph S. Beckman, Victor M. Darley-Usmar, and Hanjoong Jo. Evidence for peroxynitrite as a signaling molecule in flow-dependent activation of c-Jun NH2-terminal kinase. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1647–H1653, 1999.—The c-Jun NH2-terminal kinase (JNK), also known as stress-activated protein kinase, is a mitogen-activated protein kinase that determines cell survival in response to environmental stress. Activation of JNK involves redox-sensitive mechanisms and physiological stimuli such as shear stress, the dragging force generated by blood flow over the endothelium. Laminar shear stress has antiatherogenic properties and controls structure and function of endothelial cells by mechanisms including production of nitric oxide (NO) and superoxide (O2). Here we show that both NO and O2 are required for activation of JNK by shear stress in endothelial cells. The present study also demonstrates that exposure of endothelial cells to shear stress increases tyrosine nitration, a marker of reactive nitrogen species formation. Furthermore, inhibitors or scavengers of NO, O2, or reactive nitrogen species prevented shear-dependent increase in tyrosine nitration and activation of JNK. Peroxynitrite alone, added to cells as a bolus or generated over 60 min by 3-morpholinosydnonimine, also activates JNK. These results suggest that reactive nitrogen species, in this case most likely peroxynitrite, act as signaling molecules in the mechanostimulation of JNK.

nitric oxide synthase; NAD(P)H oxidase; superoxide dismutase; manganese(III)tetrakis(4-benzoic acid) porphyrin

Cells respond to mechanical forces such as stretch and shear stress by producing superoxide (O2) or hydrogen peroxide (H2O2) through regulated pathways (2, 6, 42). However, it is not clear whether these reactive species are simply metabolic by-products or serve some other, more specific, function. The sustained exposure of the endothelium to laminar shear stress is associated with antiatherogenic responses (5, 46) and also stimulates production of nitric oxide (NO) and reactive oxygen species (ROS) (2, 6, 16, 19, 21). The production of NO and O2 has been demonstrated in response to flow in a variety of experimental models including intact blood vessels and cultured endothelial cells (21). It is therefore important to understand shear stress-dependent signaling from both the perspective of atherosclerosis and the molecular basis of redox-dependent signal transduction.

Shear stress controls vascular function by orchestrating the production of vasoactive factors and other endothelial responses including gene transcription (5). Important genes regulated by shear stress include endothelial nitric oxide synthase (eNOS), both the Cu/Zn and Mn superoxide dismutases (SOD) (36), and other modulators of vascular function (5). Induction of specific genes by shear stress in endothelial cells is mediated by activation of mechanosensitive signaling pathways that include at least three members of the mitogen-activated protein (MAP) kinase family, extracellular signal-regulated kinases 1 and 2 (ERK), c-Jun NH2-terminal kinase (JNK), and Big MAP kinase 1 (11, 14, 15, 22, 38, 44). MAP kinases are important signaling components linking extracellular stimuli to cellular responses such as cell growth, death, differentiation, and metabolic regulation (3, 17).

Shear stress activates ERK in a rapid and transient manner (maximum activation by 5 min and returning to basal levels by 30 min of shear exposure), whereas JNK activation occurs over a much slower and prolonged time course (requiring at least 30 min and returning to basal levels after 1 day of shear exposure) (15). Current evidence also indicates that shear stress activates two MAP kinases by distinct signaling pathways: activation of ERK is mediated by mechanisms involving Gtα2, protein kinases [Src, focal adhesion kinase (FAK), and protein kinase Cε], and Ras, whereas JNK activation requires Gtβ/γ, phosphatidylinositol 3-kinase-γ, tyrosine kinases (Src and FAK), and Ras (11, 14, 15, 22, 37). Additional evidence suggests that a specialized signaling domain in the plasma membrane, called caveolae, plays an essential role in the selective activation of the shear-dependent ERK pathway (29, 30).

Recent evidence suggests that NO and ROS can activate MAP kinases in Jurkat T cells, HEK293 cells, and chondrocytes (18, 20, 24). However, it is not known whether NO and ROS also regulate activation of MAP kinases in response to the physiological stimulation of laminar shear stress. An important function of NO is...
modulation of vascular tone through relaxation of smooth muscle cells (27), but little is known about the effects of the reaction products of NO with ROS on the endothelium. This raises interesting questions about the potential significance of the simultaneous formation of NO and O$_2^-$ in the endothelium, which, in other contexts, is generally viewed as being deleterious and cytotoxic (4). However, laminar shear stress is not cytotoxic to endothelial cells and, in fact, protects against cytokine-induced apoptosis (7). JNK activation plays a dual role in either promoting or protecting against cell death, depending on the cell type and its environment (23, 43, 45). Because the production of reactive oxygen and nitrogen species often occurs in pathological conditions, the detailed understanding of how this MAP kinase responds to reactive species is essential. These observations led to the hypothesis that, at low concentrations (in the nanomolar range), the combined effects of NO and O$_2^-$ produced in response to shear stress and their associated nitrating and/or nitrosating properties play a role in cell signaling. This hypothesis was investigated in the context of the shear-dependent activation of JNK in endothelial cells.

**MATERIALS AND METHODS**

Cell culture, transfection protocols, and shear stress. Endothelial cells obtained from bovine thoracic aortas (BAEC) were used between passages 5 and 10 and were prepared for shear experiments by seeding 1 x 10$^6$ cells onto glass slides (75 x 38 mm) as described previously (15). Plasmids encoding for hemagglutinin-tagged JNK1 (HA-JNK1) and c-jun (amino acids 5–89) fused to glutathione S-transferase (GST-c-jun) were described previously (15). Cu/Zn SOD in pET-3d vector was subcloned into a BamH I/Xba I site of pcDNA3.1 vector containing an Myc epitope in the COOH terminus (Invitrogen), and the DNA was sequenced. The transfection method using adenovirus conjugated to poly-L-lysine and the techniques used to expose cells to laminar shear stress with the use of a parallel-plate shear chamber and flow loop have been described elsewhere (15).

MAP kinase assays. After shear exposure, cell lysates were obtained and ERK activation in the cell lysates (10 µg) was examined by Western blot analysis with the use of an antibody specific to the active, phosphorylated form of ERK (phospho-ERK) (New England Biolabs) as described previously (15). The JNK assay was carried out by using an antibody specific for JNK1 (Pharmingen) or HA epitope (Boehringer Mannheim). These were incubated with the soluble lysates (100 µg) for 1 h at 4°C, followed by an additional 1-h incubation with Protein G-agarose beads. The immune complex was washed and incubated in the presence of GST-c-jun and [32P]JATP as described (15). The reaction products were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane, the autoradiogram was obtained, and the radioactivity incorporated into each band was quantified by scintillation counting. The membrane was then probed with a polyclonal antibody to JNK to monitor the total amount of immunoprecipitated JNK in each experiment.

Immunohistochemical staining with a nitrotyrosine antibody. After treatments, cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde for 30 min, permeabilized in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 50 mM lysine for 30 min, and blocked in PBS containing 2% BSA, 10% goat serum, and 0.1% Triton X-100 for 1 h. Cells were then incubated with a polyclonal nitrotyrosine antibody overnight at 4°C and with Cy3-conjugated goat anti-rabbit for 30 min. Washed cells were then mounted with Slow-Fade, and fluorescence intensity was quantified by using a fluorescence microscope and an image analysis program (ESPRIT program, Olympus) (9).

Measurement of NO release. Because >90% of NO released is measured as nitrite, formation of NO was monitored by measuring nitrite released into the medium during shear stress (10 dyn/cm$^2$) or static control as described previously (40).

Treatment of BAEC with peroxynitrite. Peroxynitrite was synthesized as described previously (41). Confluent BAEC monolayers were exposed to ONOO$^-$ or decomposed ONOO$^-$ in PBS for 1 min, and the reaction with dihydroxydihydronicotinic acid was used to determine exposure of the cells to the oxidant (8, 41). With the use of this protocol, 50–500 µM authentic ONOO$^-$ was added to the buffer under conditions identical to those used to study the effect of ONOO$^-$ on JNK activation in BAEC resulted in oxidation of 5–50 µM dihydroxydihydronicotinic acid, similar to results in the literature (8). The period of exposure of cells to ONOO$^-$ was calculated from its rate of decomposition under these conditions (0.65 s$^{-1}$) and was 2–3 s (4, 41). Thus, after this period, ONOO$^-$ was essentially decomposed, and after 1 min, cells were returned to normal medium. To expose cells to a similar amount of ONOO$^-$ for a sustained period, a concentration of 3-morpholinosydnonimine (SIN-1; 500 µM) was selected to generate ONOO$^-$ over 1 h (10, 12). Again, ONOO$^-$ formation was assessed by measuring the oxidation of dihydroxydihydronicotinic acid under identical conditions (41). To calculate the steady-state exposure of the cells to ONOO$^-$ generated from SIN-1, we simulated the reaction, taking into account the effects of carbon dioxide (2 mM) and the measured decomposition rate of ONOO$^-$ in this cell culture medium (0.45 s$^{-1}$) (25).

**RESULTS**

To determine whether NO plays an essential role in MAP kinase activation, the effects of NOS inhibitors on the shear-dependent activation of ERK and JNK were determined. As shown previously (15), exposure of BAEC shear stress (10 dyn/cm$^2$) for 5 min stimulated activity of ERK, whereas activation of J NK required 30–60 min (Fig. 1). Treatment of BAEC with the NOS inhibitor N$^g$-nitro-$l$-arginine methyl ester ($l$-NAME) had no effect on basal or shear-dependent activity of ERK (Fig. 1A). In contrast, $l$-NAME completely blocked shear-dependent activation of JNK (Fig. 1B) and NO release. Nitrite accumulation in the medium decreasing from 1.99 ± 0.7 mmol/mg protein in cells exposed to shear stress for 30 min to 0.23 ± 0.13 mmol/mg protein in the presence of $l$-NAME. Similar effects on MAP kinase activation were obtained using other NOS inhibitors including $N^g$-nitro-$l$-arginine and $N^g$-monomethyl-$l$-arginine citrate (Fig. 1C). These results show the essential and selective role that NO plays in the signaling pathways leading to activation of JNK by shear stress.

To determine whether NO alone and the classic NO-sensitive soluble guanylate cyclase pathway could activate JNK, we treated cells with two different NO donors, S-nitrosopenicillamine and diethylengleta-
amine-NONOate, or a cell-permeable cGMP analog, 8-(4-chlorophenylthio)-cGMP (8-CPT-cGMP). Rates of release of NO from the two NO donors (200 µM) were determined in a separate experiment and were found to be 1–4 nM/s, which is similar to the level of NO produced in endothelial cells subjected to shear stress (16). At these rates of NO release, NO donors alone had no effect on JNK activation (Fig. 2A). Treatment of BAEC with 8-CPT-cGMP (100–500 µM for ≤1 h) also had no effect on JNK activity (Fig. 2B). In the same study, activation of JNK was induced by ultraviolet irradiation in BAEC as a positive control (Fig. 2B). Furthermore, inhibition of soluble guanylate cyclase by treating the cells with 3 µM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one had no effect on basal or shear-dependent activation of JNK (data not shown). These results indicate that the cGMP pathway does not play an essential role in shear activation of JNK. The results shown thus far suggest that shear-dependent activation of JNK requires NO, but NO alone is not sufficient, and additional factors are necessary to activate this MAP kinase.

Shear stress stimulates not only NO production (16,19) but also ROS formation including O$_2^-$ (2, 6, 21). The rate of reaction between NO and O$_2^-$ is diffusion limited (4), suggesting the potential for an interactive effect of these radicals in flow-mediated JNK activation. Initially, the effects of two inhibitors of potential sources of O$_2^-$ or H$_2$O$_2$ in the cell were examined. Cells were treated with apocynin [an inhibitor of NAD(P)H oxidase] (1) or allopurinol (an inhibitor of xanthine oxidase) before being subjected to shear stress. Apocynin partially inhibited shear-dependent activation of JNK, whereas allopurinol had no effect (Fig. 3). The partial effect of apocynin could be due to production of O$_2^-$ from additional sources including mitochondria or even NOS (39). Because there are no specific inhibitors of mitochondrial O$_2^-$ formation, we cannot exclude its
potential role in this JNK pathway. However, a contribution of NOS can be addressed because all the NOS inhibitors used (Fig. 1) completely prevent NO formation while having variable but relatively little effect on O$_2^-$ (39). These data indicate that NOS is not a significant source of O$_2^-$ for JNK activation in response to shear.

Two further approaches were taken to test for a role of O$_2^-$ in JNK activation. First, an SOD mimetic, manganese(III)tetrakis(4-benzoic acid) porphyrin (Mn-TBAP), was found to inhibit flow-dependent JNK activation in a concentration-dependent manner (Fig. 4A). This metalloporphyrin is not totally specific for O$_2^-$ because it may also catalyze the decomposition of ONOO$^-$ (35). As a further test for the role of O$_2^-$, cells were cotransfected with the enzyme Cu/Zn SOD (tagged with c-Myc epitope) and HA-tagged JNK (Fig. 4B, top) by using a method of adenovirus conjugated to poly-L-lysine as described previously (15). Although cells were transfected at 20–30% transfection efficiency using this method, the effect of overexpressing Cu/Zn SOD on JNK activity can be specifically studied by cotransferring cells with HA-JNK and by subsequent immune kinase assay of the HA-tagged JNK (15). The expression of recombinant Cu/Zn SOD was confirmed by Western blot analysis using a c-Myc antibody (Fig. 5A). The transient overexpression of Cu/Zn SOD prevented shear-dependent activation of JNK (Fig. 4B), implying that O$_2^-$ plays a critical role in this process. Because endothelial cells have an endogenous level of SOD, these data suggest that control of the activity of this enzyme determines the threshold for the NO/O$_2^-$-dependent activation of JNK.

The dismutation product of O$_2^-$ is H$_2$O$_2$, which has been shown to activate JNK when added directly to cells (24). However, H$_2$O$_2$ is not likely to be playing a significant role in the responses described here for a number of reasons. Most importantly, the rate of reaction of NO and O$_2^-$ is sufficiently rapid to theoretically compete with dismutation by SOD in the cell. Thus SOD transfection should either have no effect on H$_2$O$_2$ steady-state levels or could lead to an increase. In either case, if H$_2$O$_2$ were responsible for activation of JNK, SOD overexpression should not have prevented flow-dependent stimulation of the MAP kinase. Taken together, these data indicate a requirement for both NO and O$_2^-$ formation in activation of this signaling pathway. This could occur by separate and independent actions of NO and O$_2^-$ or as a consequence of the complex reactions, including ONOO$^-$ formation, that occur between these two radicals.

Reactive nitrogen species can react with biomolecules to form stable adducts such as nitrotyrosine that can be detected by immunohistochemistry (9). The evidence thus far reveals that reactive nitrogen species derived from NO and O$_2^-$ may be formed in response to shear stress, which in turn could lead to formation of nitrotyrosine. With the use of an antibody specific to nitrotyrosine, a two- to threefold increase in shear stress-dependent nitrotyrosine staining was observed in BAEC (Fig. 5, A and B). Excess free nitrotyrosine inhibited the shear-dependent increase in immunofluorescence, indicating specific binding to nitrotyrosine by the antibody (Fig. 5C). Pretreatment of BAEC with a concentration of Mn-TBAP that inhibited JNK activation (Fig. 4A) also blocked the shear-dependent increase in the intensity of nitrotyrosine staining (Fig. 5D), consistent with the reported scavenging effects of this porphyrin toward ONOO$^-$ (31). These data suggest that reactive nitrogen species, possibly ONOO$^-$, are formed during shear stress and mediate shear-dependent activation of JNK. The role of tyrosine nitration in the signal transduction mechanisms leading to JNK activation are not yet known.

To determine whether ONOO$^-$ alone can activate JNK, we incubated BAEC with either the chemically synthesized, authentic ONOO$^-$ or a compound that generates NO and O$_2^-$ at comparable rates, SIN-1 (10,
12). Bolus additions of authentic ONOO$^{-}$ (50–500 $\mu$M) to BAEC resulted in an exposure of the oxidant to the cells that lasted ~2–3 s and was estimated to be equivalent to 5–50 $\mu$M as assessed by dihydrorhodamine oxidation (8, 41). This treatment stimulated JNK activity in a concentration-dependent manner, whereas the decomposition products of ONOO$^{-}$ had no effect (Fig. 6A). Despite the short exposure, significant activation of JNK occurred in response to the bolus addition of ONOO$^{-}$ 30–60 min after the initial addition (data not shown). Because shear-dependent production of ONOO$^{-}$ is likely to be a cumulative process, dependent on the relative fluxes of NO and O$_2^-$, bolus addition of the oxidant may not elicit the same responses as low-dose exposure over a longer time period. This possibility was tested by exposing BAEC for 1 h to the compound SIN-1, which releases both NO and O$_2^-$ (10). The results of this experiment are shown in Fig. 6B and indicate that JNK activation by SIN-1 occurs with production of the oxidant at a rate of 4–8 nM/s over the 1-h period. As a cumulative exposure of ~15–30 $\mu$M ONOO$^{-}$, this achieves a level of JNK activation similar to that of bolus addition of preformed ONOO$^{-}$ (Fig. 6). In cells SIN-1 may produce somewhat more NO than O$_2^-$ due to the availability of alternative electron acceptors for metabolism; thus ONOO$^{-}$ fluxes may, in fact, be overestimated (32). However, the data shown in Fig. 2 indicate that NO alone cannot activate JNK under these conditions.

**DISCUSSION**

These results place a new perspective on the significance of the reaction of NO and O$_2^-$ in a biological setting. Previous studies using NO-specific probes have measured NO release (1–4 nM/s) from endothelial cells subjected to shear (16). In biologic systems it is exceedingly unlikely that a 1:1 stoichiometry of NO and O$_2^-$ occurs except for transient periods. The data further indicate that ONOO$^{-}$ generated as a constant flux or added in its chemical form can activate JNK, although the concentrations required are somewhat higher, approximately 10-fold, than that likely to be occurring in response to shear stress. There are a number of reasons that could account for such differences, including 1) the selective advantage of producing ONOO$^{-}$ as a signaling molecule within the cell and/or 2) the possibility that shear stress activates convergent signaling pathways leading to a more sensitive response to ONOO$^{-}$. This is important, because in local subcellular compartments ONOO$^{-}$ may mediate specific modification of signaling proteins at thiol or aromatic amino acid residues. For example, nitrosation of thiols forms S-nitrosothiols, and their potential as transducers of cell signaling events has been recognized for some time (7, 26, 33). Furthermore, S-nitrosothiols are indeed released from endothelial cells in response to shear stress (34). It is postulated that a reactive species, capable of mediating nitration or nitrosation reactions, can exhibit specific-
ity required for activation of cell signaling pathways. The basis of selectivity may lie in the detailed upstream events in the JNK signaling pathway, some element of which can be sensitive to modification by reactive nitrogen species. For example, JNK activation is mediated by Ras-dependent mechanisms (15), and it is known that Ras activity can be modulated by S-nitrosation of a specific cysteine residue (20). It is interesting, however, that inhibitors of NOS, xanthine oxidase, or NAD(P)H oxidase did not show any effect on shear-dependent activation of ERK (data not shown), even though this MAP kinase pathway is also regulated by Ras (15). Recent evidence suggests that this differential signaling specificity is conferred by spatial sequestration of signaling molecules into intracellular microcompartments such as caveolae (29, 30). By inference, this also suggests that redox signaling mechanisms also can be compartmentalized.

In summary, the current study has shown that a physiological stimulus, shear stress, induces reactive nitrogen species production in endothelial cells. Furthermore, preventing the formation of reactive nitrogen species leads to the inhibition of the mechanosensitive JNK activation, thus suggesting that reactive nitrogen species are an essential mediator of this signaling pathway in endothelial cells. As with a number of second messengers, including NO, biologic effects are critically dependent on concentration. Because the maximal production of ONOO\textsuperscript{−}, or any mediator derived from these reactions, in response to shear stress is constrained by the formation of NO, the concentrations of the signaling molecule can only be in the nanomolar range (16). Recent evidence has shown that low concentrations (nanomolar to low micromolar) of ONOO\textsuperscript{−} play a cardioprotective role by inhibiting P-selectin expression and leukocyte-endothelium interaction in ischemia-reperfused myocardium (28). We now propose that low levels of ONOO\textsuperscript{−} regulate specific signaling pathways.

Whereas these studies provide compelling evidence that both NO- and O\textsubscript{2}•−-dependent mechanisms are required for JNK activation, the molecular basis underlying this interaction remains to be determined. There are at least two possibilities: 1) NO and O\textsubscript{2}•− activate two independent pathways that converge upstream to activate JNK; or 2) the reaction product of NO and O\textsubscript{2}•−, ONOO\textsuperscript{−}, itself activates a specific signaling component in the JNK pathway. Full elucidation of these aspects requires the use of pharmacologically specific scavengers of ONOO\textsuperscript{−} that have yet to be developed. Metalloporphyrins are promising in this respect, and indeed some have argued that in a cellular context Mn-TBAP behaves as a peroxynitrite decomposition catalyst (13, 31).

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REFERENCES
18. Kim, H., J. Shim, P. L. Han, and E. J. Choi. Nitric oxide modulates the c-jun N-terminal kinase/stress-activated protein...


