Involvement of the mitogen-activated protein kinase cascade in peroxynitrite-mediated arachidonic acid release in vascular smooth muscle cells

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Involvement of the mitogen-activated protein kinase cascade in peroxynitrite-mediated arachidonic acid release in vascular smooth muscle cells. Am. J. Physiol. Cell Physiol. 286:C1271–C1280, 2004. First published January 28, 2004; 10.1152/ajpcell.00143.2003.—Eicosanoid production is reduced when the nitric oxide (NO) pathway is inhibited or when the inducible NO synthase gene is deleted, indicating that the NO- and arachidonic acid pathways are linked. We hypothesized that peroxynitrite, formed by the reaction of NO and superoxide anion, may cause signaling events leading to arachidonic acid release and subsequent eicosanoid generation. Western blot analysis of rat arterial smooth muscle cells demonstrated that peroxynitrite (100–500 μM) and 3-morpholinosydnonimine (SIN-1; 200 μM) stimulate phosphorylation of extracellular signal-regulated kinase (ERK), p38, and cytosolic phospholipase A2 (cPLA2). We found that peroxynitrite-induced arachidonic acid release was completely abrogated by the mitogen-activated protein/ERK kinase (MEK) inhibitor U0126 and by calcium chelators. With the p38 inhibitor SB-20219, we demonstrated that peroxynitrite-induced p38 phosphorylation led to minor arachidonic acid release, whereas U0126 completely blocked p38 phosphorylation. Addition of arachidonic acid caused p38 phosphorylation, suggesting that arachidonic acid or its metabolites are responsible for p38 activation. KN-93, a specific inhibitor of Ca2⁺/calmodulin-dependent kinase II (CaMKII), revealed no role for this kinase in peroxynitrite-induced arachidonic acid release in our cell system. Together, these results show that in response to peroxynitrite the cell initiates the MEK/ERK cascade leading to cPLA2 activation and arachidonic acid release. Thus studies investigating the role of the NO- pathway on eicosanoid production must consider the contribution of signaling pathways initiated by reactive nitrogen species. These findings may provide evidence for a new role of peroxynitrite as an important reactive nitrogen species in vascular disease.

reactive nitrogen species; prostaglandin H₂ synthase; extracellular signal-regulated kinase; p38; cytosolic phospholipase A₂

REACTIVE NITROGEN SPECIES (RNS), such as nitric oxide (NO⁻) and peroxynitrite (ONOO⁻), play an important role in host defense mechanisms. However, when released in an unrestrained manner, they may become involved in the pathogenesis of several inflammatory diseases that include rheumatoid arthritis (31) and atherosclerosis (5, 13, 64). These diseases involve arachidonic acid metabolites. One route of arachidonic acid metabolism, which results in eicosanoid generation, involves the constitutive and inducible forms of prostaglandin H₂ synthase (PGHS-1 and PGHS-2, respectively). A disruption in the balance of essential eicosanoids is thought to contribute to the pathophysiology of inflammatory vascular disease (11). Because RNS and eicosanoids are often found at sites of inflammation, where their synthesis is often elicited by the same stimuli, an understanding of the interactions between RNS and the arachidonic acid pathway is of great interest. Thus the determination of those processes involving RNS that contribute to changes in eicosanoid levels during inflammatory disease is of clinical importance.

There is now strong evidence linking the NO and arachidonic acid pathways. For instance, mice bearing a targeted deletion in the inducible NO synthase (iNOS) gene demonstrate reduced ability to synthesize prostaglandin E₂ (PGE₂) (42). Similarly, administration of NOS inhibitors to rats leads to a reduction in prostaglandin production in inflammatory lesions (56, 57). Furthermore, inflamed brain tissue from iNOS-knockout mice reveals decreased levels of PGE₂ compared with wild-type mice (46, 54). In addition, arginine depletion can decrease prostaglandin synthesis in mouse macrophages stimulated with lipopolysaccharide (LPS) (54, 55). These observations indicate that NO and/or NO-derived species can alter PGHS activity and eicosanoid production in vivo.

The chemistry of NO- under physiological conditions is complex, leading to the formation of various RNS that include NO⁻, nitrosonium ion (NO⁺), nitroxyl anion (NO⁻), nitrite (NO₂⁻), nitrate (NO₃⁻), peroxynitrite (ONOO⁻) (62) and nitrosoperoxycarbonate (ONOOCO₃⁻) (50). For this reason, the type of interaction between RNS and PGHS enzymes is highly dependent on the nature of the species under consideration. For example, ONOO⁻, which arises from the reaction between NO⁻ and superoxide radical anion (27), activates PGHS (34, 63), whereas NO⁻ inhibits activation (63). The mechanism of ONOO⁻-induced PGHS activation is likely similar to that of peroxide activation of PGHS, which involves oxidation of the heme moiety (15). ONOO⁻ has also been shown to cause Tyr nitration in PGHS in vascular smooth muscle cells (13) and in platelets (7). These results indicate that it is possible for a specific RNS such as ONOO⁻ to have both an inhibitory and activating effect.

Although RNS directly affect the activity of PGHS enzymes, the effect of RNS on the signaling pathways that lead to arachidonic acid release and increased PGHS activity has not been fully investigated. Arachidonic acid release from arachidonyl phospholipids is catalyzed by cytosolic phospholipase

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A2 (cPLA2) (9, 37). cPLA2 activation, which requires both phosphorylation and an increase in intracellular Ca2+ (25, 37), is achieved by several agonists, including growth factors, neurotransmitters, angiotensin II, vasopressin, LPS, colony-stimulating factor-1, and thrombin (32, 37, 38). cPLA2 is phosphorylated on Ser505 in vitro by mitogen-activated protein (MAP) kinases (MAPKs), including the extracellular signal-regulated kinases (ERKs) and p38 MAPK (32, 39, 45, 66), although the specific signaling mechanism varies in response to different agonists in different cell types. In addition, cPLA2 is phosphorylated on Ser727 by MAPK-interacting kinase I (MKI-I), or a closely related isoform (24), and on Ser515 by calcium/calmodulin-dependent protein kinase II (CaMKII) (44). When expressed in a baculovirus/Sf9 cell system, cPLA2 is phosphorylated on four serine residues (Ser437, Ser454, Ser505, and Ser727) (12). However, the signaling mechanisms by which RNS might activate cPLA2 are not yet fully elucidated.

The actions of RNS, such as ONOO−, which is known to initiate lipid peroxidation (49), react with sulfhydryl groups (48), and cause oxidative damage via Tyr nitration (13, 28), are not limited to immediate chemical damage. ONOO− has been found to activate calcium-dependent PKC, the ERKs, c-Jun NH2-terminal kinase (JNK), and p38 MAPK (3, 21, 29, 58). These kinases (Beverly, MA) were found to activate calcium-dependent PKC, the ERKs, c-Jun NH2-terminal kinase (JNK), and p38 MAPK (3, 21, 29, 58).

ONOO− also affects cellular signaling by causing dimerization of the epidermal growth factor receptor (EGFR) in PC-12 cells, possibly through intermolecular dityrosine cross-linking (65), but may also occur independently of the EGFR receptor (71). It has been reported that ONOO− causes the release of arachidonic acid from PC-12 cells (23) due to stimulation of a low-molecular-mass form of cPLA2 in mitochondria isolated from these cells (22). However, the specific role of ONOO− in the signaling cascade leading to cPLA2 activation and arachidonic acid release in vascular smooth muscle cells has not yet been examined.

In this study, we found that arachidonic acid is released in vascular smooth muscle cells after challenge with ONOO−. To investigate the hypothesis that ONOO− stimulates the phosphorylation of signaling molecules leading to the activation of cPLA2, we examined the effect of ONOO− on the p38 MAPK, CaMKII, and ERK signaling pathways. We believe our new findings define the contributions of specific signaling pathways linking ONOO− to changes in eicosanoid metabolism in vascular cells.

EXPERIMENTAL PROCEDURES

Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were obtained from GibCO-BRL. Sodium peroxynitrite (110–200 mM in 47% sodium hydroxide) and 3-morpholinosydnonimine (SNAP) were obtained from Calbiochem and Alexis Biochemicals, respectively. Phosphospecific polyclonal antibodies to the activated forms of MEK1/2 (Ser217/221), ERK1/2 (Thr202/Tyr204), p38 MAPK (Thr180/Tyr182), cPLA2 (Ser505), Raf-1 (Ser259), and MKK3/6 (Ser189/207) were obtained from Cell Signaling Technologies (Beverly, MA). Antibodies specific to the nonphosphorylated forms of these signaling molecules were also obtained from Cell Signaling Technologies. The polyclonal antibody specific for phosphorylated CaMKII (Thr287) was obtained from Promega. Horseradish peroxidase-conjugated antirabbit antibodies were purchased from Amersham International (Arlington Heights, IL). 4-4’(Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole (SB-202190), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), KN-93, and 1,2-bis(2-aminophenoxymethylene)-N,N,N’,N’-tetraacetic acid tetrakis (acetoxyethyl) ester (BAPTA-AM) were obtained from Calbiochem. Ethylene glycol bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA) was obtained from J. T. Baker. 3-H-Labeled arachidonic acid (100 Ci/mmol) was purchased from New England Nuclear (Boston, MA), whereas nonlabeled arachidonic acid was obtained from Aldrich. All the materials for SDS-PAGE were obtained from Bio-Rad Laboratories (Hercules, CA).

UV/Vis spectroscopy and handling of ONOO−. The concentration of ONOO− was determined with a Perkin Elmer UV/Vis spectrophotometer by measuring its absorbance at 302 nm (ε = 1,670 M−1·cm−1). Matching quartz precision cells (Hellma, 1-cm path length; 1-ml or 100-μl volume) were used in our measurements. On receiving ONOO−, the sample was thawed, its concentration was determined by UV/Vis spectroscopy, and it was divided into smaller aliquots, snap frozen, and stored at −80°C until required. During an experiment, an aliquot of ONOO− was kept on dry ice until its addition was required, at which point it was thawed and added directly to the well. The remnants of the sample were immediately snap frozen on dry ice, and the concentration of the sample was determined after completion of the experiment. A comparison of the ONOO− concentration before and after the experiment allowed us to determine the quality and consistency of ONOO− used in the experiment. These protocols ensured reproducibility. Aliquots used in an experiment were not reused.

Smooth muscle cell culture. Primary passage rat smooth muscle cells were isolated and characterized as described previously (47). Cells were plated either in six-well dishes (for arachidonic acid release or PGE2/6-keto-PGF1α measurements in the supernatant) or in 100-mm dishes (for the measurement of phosphorylated signaling molecules in the cell lysate) in DMEM supplemented with 10% (vol/vol) fetal bovine serum and 1% (vol/vol) glutamine. All cells were incubated at 37°C in 5% CO2 in air. Cells were rendered quiescent for 3–4 days with serum-free medium (quiescent medium) containing insulin, transferrin, and selenium (ITS; Life Technologies) to suppress PGHS-2 expression (52). Nitrate/nitrite levels were measured with a colorimetric assay (Cayman Chemical) and were found to be indistinguishable from the supernatant medium of quiescent cells. Cells between passages 6 and 15 were used in experiments. We did not see significant variation in our results related to passage number.

Treatment of smooth muscle cells. Quiescent rat smooth muscle cells were exposed to either quiescent medium or quiescent medium with added ONOO− or arachidonic acid for 1 h at 37°C. In experiments requiring ONOO−, ONOO− from a stock solution (100-200 mM) was added directly to the medium in the form of a single bolus while the dish was swirled (63). This immediate addition strategy was performed because ONOO− has an extremely short half-life. In control experiments, ONOO− was allowed to decompose (as determined spectrophotometrically) at room temperature for 2–3 days. In other control experiments, cells were treated with NaOH as vehicle control (data not shown). In experiments using the inhibitors (including SB-202190, U0126, KN-93, or BAPTA-AM and EGTA) cells were first pretreated with the inhibitor (1 h for SB-202190, KN-93, BAPTA-AM, and EGTA; 40 min for U0126) and then stimulated with ONOO− (200 or 500 μM), or in some cases arachidonic acid (10 μM), for time points that ranged between 0 and 60 min, depending on the type of experiment being performed. PGE2 and 6-keto-PGF1α measurements. Quiescent smooth muscle cells in six-well dishes were exposed to ONOO− (100, 200, and 500 μM) for 1 h (or for time points between 0 and 60 min), and the supernatant medium was assayed for either PGE2 or 6-keto-PGF1α, or PGE2/6-keto-PGF1α formation with enzyme immunoassay assay kits (Amersham Pharmacia). Quiescent smooth muscle cells in six-well dishes were also exposed to 100–500 μM nitrite in vitro to assess nitroso-N-acetyl-penicillamine (SNAP), 1-hydroxy-2-oxo-3-(N-methyl-aminopropyl)-3-methyl-1-triazene (NOC-7), and SIN-1. Total protein in the cell lysate was determined by using either the Lowry or the modified Lowry method.
(41). After exposure of the cells to 100, 200, and 500 μM ONOO−, nitrate/nitrite levels in the supernatant medium were found to be 114 ± 7, 222 ± 4, and 473 ± 10 μM nitrate/nitrite, respectively, which closely reflects the expected concentration of the decomposition products of ONOO−.

Western blotting. After treatments, cells were washed with PBS and lysed in buffers, the composition of which varied depending on which phosphorylated signaling molecule was under investigation. The modified lysis buffer used for detecting the phospho-specific forms of p38, MEK1/2, ERK1/2, cPLA2, Raf-1, and MKK3/6 comprised (in mM) 20 Tris-HCl pH 7.5, 1 EDTA, 1 EGTA, 150 NaCl, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 sodium vanadate, and 1 phenylmethylsulfonyl fluoride (PMSF) with 1% Triton X-100 and 1 μg/ml leupeptin. The modified lysis buffer used for detecting the phospho-specific form of CaMKII comprised (in mM) 20 Tris-HCl pH 8, 2 EDTA, 2 EGTA, 10 sodium phosphate, 2 dithiothreitol, 25 benzamidine, and 1 PMSF with 25 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 10 μg/ml aprotinin. The cells were scraped, transferred to an Eppendorf tube, vortexed, and put on ice for 30 min. The lysates were clarified by centrifugation (10,000 g at 4°C) for 10 min. The protein sample (25–50 μg) was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blocked for 90 min at 25°C with 5% nonfat milk in a PBS-1% Tween buffer. The membrane was then incubated overnight at 4°C with an appropriate dilution of the phospho-specific antibody, followed by incubation for 75 min with a 1:2,000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody. The immunoblot signal was visualized through enhanced chemiluminescence.

Determination of arachidonic acid release. Confluent rat smooth muscle cells in six-well dishes were labeled for 24 h with [3H]arachidonic acid (0.5–1 μCi/ml) in quiescent DMEM. The cells were washed three times with quiescent DMEM containing 0.2% fatty acid-free bovine serum albumin (BSA) and once in PBS to remove all unincorporated [3H]arachidonic acid. Approximately 80–90% of the added [3H]arachidonic acid was incorporated into the cells. The cells were treated with and without inhibitors and stimulated with ONOO− as described above. Before ONOO− addition, 100 μl of the medium was removed and counted for the presence of [3H]label. After ONOO− treatment, another 100 μl of the medium was removed and counted. The difference obtained served as the measure of arachidonic acid release.

Miscellaneous. Cell viability was assessed by in situ staining with Trypan blue, and cell number was determined by hemocytometer. At 500 μM ONOO−, cell viability experiments demonstrated no significant cell death (data not shown). ONOO− becomes toxic under our experimental conditions at levels of 1 mM and higher. Unless otherwise stated, data are reported as averages ± SE, with significant differences determined by a single-factor ANOVA or by Student’s t-test.

RESULTS

ONOO− increases PG2 synthesis and arachidonic acid release in a time- and concentration-dependent manner. Figure 1A shows that, in the absence of exogenously added arachidonic acid, treatment of smooth muscle cells with ONOO− caused a dose-dependent increase in PG2 formation. This effect was also observed with SIN-1, which generates ONOO− on decomposition (Ref. 53 and data not shown). In contrast, the following had no effect on PG2 formation: nitrite (a decomposition product of ONOO−), SNAP, which contains a nitrosamine group (NO+), and NOC-7, which releases NO− and sodium hydroxide vehicle (data not shown). ONOO− was found to induce PG2 synthesis in a time-dependent manner, as shown in Fig. 1B, with maximal PG2 formation occurring in ~15 min. To examine further whether ONOO− induces cPLA2 activation, we measured [3H]-labeled arachidonic acid release in smooth muscle cells after ONOO− treatment. Figure 1C shows that arachidonic acid release in the medium increased proportionally with ONOO− concentration. In addition, arachidonic acid release occurred maximally after ~15 min following the addition of ONOO−, as shown in Fig. 1D. Formation of 6-keto-PGFα1 (which is the stable end product of prostacyclin (PGI2)) also increased in response to ONOO−, as shown in Fig. 1E.

ONOO− and SIN-1 activate signaling molecules in a time-dependent manner. Figure 2 demonstrates the temporal effect of ONOO− and SIN-1 on the signaling molecules p38, MEK1/2, ERK1/2, and cPLA2. The effect of ONOO− and SIN-1 on the activation of these molecules was studied by Western blot analysis using antibodies specific for the phosphorylated and nonphosphorylated forms of these molecules. Figure 2, A–D, shows that ONOO− and SIN-1 induced p38, MEK1/2, ERK1/2, and cPLA2 phosphorylation in a time-dependent manner. Phosphorylation in each case was observed by ~15 min. Thus ONOO− and SIN-1 activate different signaling molecules, although these results do not yet indicate whether their activation is instrumental in causing arachidonic acid release.

ONOO− activates signaling molecules in a concentration-dependent manner. Figure 3 demonstrates the dose-dependent ability of ONOO− (100–500 μM) to induce the phosphorylation of p38 (Fig. 3A), MEK1/2 (Fig. 3B), ERK1/2 (Fig. 3C) and cPLA2 (Fig. 3D). The effect of ONOO− on the activation of these signaling molecules was again studied by Western blot analysis with antibodies specific for their phosphorylated and nonphosphorylated forms. In all cases, ONOO− induced phosphorylation in a concentration-dependent manner. There was little or no response below 100 μM ONOO−.

p38 MAPK inhibitor SB-202190 partially inhibits ONOO−-dependent arachidonic acid release. To evaluate the role of p38 MAPK in the signaling pathway of ONOO−-dependent arachidonic acid release and PG2 synthesis, cells were pretreated with SB-202190 (25 μM), a specific inhibitor of p38, and then exposed to ONOO− (500 μM). Figure 4A shows that the p38 MAPK-specific inhibitor SB-202190 did not fully inhibit ONOO−-dependent arachidonic acid release (~37% inhibition) from smooth muscle cells prelabeled with [3H]arachidonic acid. This inhibition was significant (P < 0.004). These results indicate that although ONOO− induces p38 phosphorylation, the p38 MAPK pathway is not fully responsible for ONOO−-dependent arachidonic acid release. Because these results implicate p38 MAPK in ONOO−-induced arachidonic acid release, we decided to investigate the involvement of MKK3/6, which is known to activate p38 MAPK, in this process (20). Figure 4B shows that MKK3/6 is phosphorylated in the presence of ONOO− at 5 min. Thus p38 MAPK activation is likely induced by MKK3/6.

CaMKII activation by ONOO− does not lead to arachidonic acid release. Figure 5A demonstrates the temporal effect of ONOO− on the signaling molecule CaMKII, which has been shown to be involved in cPLA2 activation (43, 44). ONOO−-dependent CaMKII phosphorylation occurred maximally at 5 min but thereafter dissipated. ONOO−-dependent CaMKII phosphorylation was blocked by KN-93 (40 μM), which is a CaMKII inhibitor. Figure 5B, however, shows that KN-93 did not inhibit ONOO−-induced arachidonic acid release. Thus
ONOO\textsuperscript{−}-induced CaMKII signaling does not lead to arachidonic acid release in smooth muscle cells.

**MEK1/2 inhibitor U0126 completely abolishes ONOO\textsuperscript{−}-induced arachidonic acid release.** To determine the role of MAPK in ONOO\textsuperscript{−}-dependent PGE\textsubscript{2} synthesis, we used the MEK1/2 inhibitor U0126. Figure 6A shows that preincubation of smooth muscle cells with U0126 (40 μM) followed by stimulation with ONOO\textsuperscript{−} abolished ERK1/2 phosphorylation. These results clearly show that phosphorylation of ERK1/2 by ONOO\textsuperscript{−} requires MEK1/2. To determine whether cPLA\textsubscript{2} activation is dependent on the MEK/ERK pathway, the effect of U0126 on arachidonic acid release and cPLA\textsubscript{2} phosphorylation was studied. Arachidonic acid release was completely blocked with U0126, as shown in Fig. 6B, because there was no significant difference between cells exposed to both U0126 and ONOO\textsuperscript{−} vs. control cells. In addition, Fig. 6C shows that ONOO\textsuperscript{−}-induced cPLA\textsubscript{2} phosphorylation is completely abolished by U0126. Because Raf-1 is a known upstream activator of MEK1/2 (33), we investigated the involvement of Raf-1 in this process. Figure 6D shows that Raf-1 is phosphorylated in the presence of ONOO\textsuperscript{−} within 5 min. Together, these data demonstrate that ONOO\textsuperscript{−}-induced arachidonic acid release requires MEK1/2, ERK1/2, and cPLA\textsubscript{2} phosphorylation and that the upstream activator of these signaling molecules is likely Raf-1.

**p38 Phosphorylation is inhibited by MEK1/2 inhibitor U0126 and is induced by arachidonic acid.** In addition to inhibiting ONOO\textsuperscript{−}-induced ERK1/2 phosphorylation as shown in Fig. 6A, the MEK1/2 inhibitor U0126 also prevented ONOO\textsuperscript{−}-induced p38 phosphorylation as shown in Fig. 7A. This result indicates that a product of the MEK/ERK pathway leads to p38 activation. Conversely, the p38 inhibitor SB-203580 did not inhibit ONOO\textsuperscript{−}-induced ERK1/2 phosphorylation, indicating that ERK1/2 activation is not dependent on the p38 MAPK pathway (data not shown). To test whether this product is related to arachidonic acid or its metabolites, quiescent smooth muscle cells were incubated with arachidonic acid.
acid (10 μM) for 1 h and cell lysates were examined for p38 phosphorylation. The results in Fig. 7B, left, clearly demonstrate that arachidonic acid (or its metabolites) induces p38 phosphorylation, as previously demonstrated (30). In parallel experiments, we also tested for the involvement of the MEK/ERK pathway in arachidonic acid-induced p38 phosphorylation by again using the MEK1/2 inhibitor U0126. The results in Fig. 7B, right, show that p38 phosphorylation was not prevented by U0126. Thus the MEK/ERK pathway is not involved in arachidonic acid-induced p38 phosphorylation.

Calcium chelators BAPTA-AM and EGTA inhibit ONOO⁻/H₂O₂-induced arachidonic acid release. To examine whether ONOO⁻/H₂O₂-dependent arachidonic acid release is mediated by cPLA₂, which is known to require Ca²⁺ for its activity,
incubated smooth muscle cells with the membrane-permeant calcium chelator BAPTA-AM along with EGTA for 1 h followed by stimulation with ONOO\(^{-}\). Figure 8 shows that the calcium chelators decreased ONOO\(^{-}\)-induced arachidonic acid release to a level similar to that observed in control cells. These results indicate that a calcium-dependent form of PLA\(_2\) is involved in ONOO\(^{-}\)-dependent arachidonic acid release.

**DISCUSSION**

It was previously demonstrated that in the presence of arachidonic acid, ONOO\(^{-}\) enhances purified PGHS activity and also stimulates PGE\(_2\) production in arterial smooth muscle cells (7, 34, 63). Under these conditions, ONOO\(^{-}\) most likely activates the PGHS isozymes in a manner similar to peroxide activation of PGHS, involving heme oxidation (15). We now demonstrate for the first time that in vascular smooth muscle cells a separate mechanism exists whereby the presence of ONOO\(^{-}\) leads to arachidonic acid release and subsequent eicosanoid production (Fig. 1). An essential clue to the mechanism of its action is the observation that maximal PGE\(_2\) production occurs after ONOO\(^{-}\) addition after \(~15\) min. In contrast, eicosanoid synthesis after the addition of exogenous arachidonic acid is known to be complete within 1 min of PGHS-1 activation (35). The sole addition of ONOO\(^{-}\) appears to induce a signaling cascade, thus accounting for the delayed release of arachidonic acid and subsequent synthesis of PGE\(_2\) and 6-keto-PGF\(_{1\alpha}\). The observation that PGE\(_2\) and 6-keto-PGF\(_{1\alpha}\) production is increased after ONOO\(^{-}\) exposure is important because several studies have observed a link between the NO\(_\alpha\)- and arachidonic acid pathways (42, 46, 54–57). Increased eicosanoid production in the presence of functional NOS enzymes may be occurring, in part, by an ONOO\(^{-}\)-induced signaling mechanism that results in arachidonic release. The fact that we observed an enhancement of 6-keto-

\[ \text{(500 \mu M)} \]

**Fig. 5.** The Ca\(^{2+}\)/calmodulin-dependent kinase (CaMKII) inhibitor KN-93 does not prevent ONOO\(^{-}\)-induced arachidonic acid release. A: quiescent smooth muscle cells were treated with ONOO\(^{-}\) (500 \mu M) for 1 h with and without preincubation with KN-93 (40 \mu M) for 1 h. Cell lysates were subjected to Western blotting with an antibody specific for phospho-CaMKII. B: [\(^{3}\)H]arachidonic acid-labeled quiescent smooth muscle cells were pretreated with KN-93 (40 \mu M) for 1 h, followed by incubation with ONOO\(^{-}\) (500 \mu M) for 1 h. The supernatant medium was then assayed for radioactivity. [\(^{3}\)H]arachidonic acid release is expressed as dpm/ml supernatant. The results represent an average of averages obtained from 4 separate experiments in which 3–6 measurements were made per experiment. Statistical analysis (Student’s t-test) revealed that KN-93 did not significantly reduce ONOO\(^{-}\)-induced arachidonic acid release.

**Fig. 6.** The MEK1/2 inhibitor U0126 prevents ONOO\(^{-}\)-induced arachidonic acid release and blocks cPLA\(_2\) phosphorylation. A: quiescent smooth muscle cells were preincubated with and without U0126 (40 \mu M) for 40 min and then exposed to ONOO\(^{-}\) (200 \mu M) for 1 h. Cell lysates were subjected to Western blotting with antibodies specific for phospho-ERK1/2. B: [\(^{3}\)H]arachidonic acid-labeled quiescent smooth muscle cells were pretreated with U0126 (40 \mu M) for 40 min, followed by incubation with ONOO\(^{-}\) (500 \mu M) for 1 h. The supernatant medium was then assayed for radioactivity. [\(^{3}\)H]arachidonic acid release is expressed as dpm/ml supernatant. The results represent an average of averages obtained from 3 separate experiments with 3–6 measurements per experiment. Statistical analysis (Student’s t-test) revealed that U0126 significantly reduced ONOO\(^{-}\)-induced arachidonic acid release (\(P < 0.001\)). There was no significant difference in cells exposed to U0126 + ONOO\(^{-}\) vs. control cells. C: quiescent smooth muscle cells were preincubated with and without U0126 (40 \mu M) for 40 min and exposed to ONOO\(^{-}\) (200 \mu M) for 1 h. Cell lysates were subjected to Western blotting with antibodies specific to phospho-cPLA\(_2\) and cPLA\(_2\). D: quiescent smooth muscle cells were exposed to ONOO\(^{-}\) (500 \mu M) for 1 h. Cell lysates were subjected to Western blotting with an antibody specific for phospho-Raf-1. Results shown are representative of 3 independent experiments.
To determine which MAPK is responsible for the ONOO\textsuperscript{−}-mediated activation of cPLA\textsubscript{2}, we used specific inhibitors aimed at blocking either MEK1/2 or p38 MAPK. The highly specific MEK1/2 inhibitor U0126 served to prevent ERK1/2 phosphorylation (14), whereas SB-202190 specifically inhibited p38 MAPK (36). On incubation with the MEK-1/2 inhibitor U0126, ONOO\textsuperscript{−}-induced arachidonic acid release and cPLA\textsubscript{2} phosphorylation were significantly reduced to control levels, indicating full inhibition (Fig. 6). Upstream activation of ERK1/2 and MEK1/2 likely involves Raf-1, which is the main effector recruited by GTP-bound Ras (2). In these studies, we have demonstrated that Raf-1 is also phosphorylated after ONOO\textsuperscript{−} incubation. These findings suggest its involvement in the activation of the ERK-MAPK pathway. The initial signal leading to the activation of the Raf-1/MEK/ERK pathway, however, remains unknown. ONOO\textsuperscript{−} is known to permeate the membrane (1), where it could cause chemical modification of specific molecules that, in turn, initiate phosphorylation of signaling molecules. Use of the p38 inhibitor SB-202190 also led to a significant reduction in ONOO\textsuperscript{−}-induced arachidonic acid release, although only partial reduction was attained, indicating incomplete involvement of the p38 MAPK pathway (Fig. 4). p38 MAPK phosphorylation is mediated by the upstream signaling kinases MKK6/MKK3, which were also phosphorylated after ONOO\textsuperscript{−} incubation. p38 MAPK is known to participate in signal transduction initiated by cellular stress (10, 68), and thus it is reasonable to assume that ONOO\textsuperscript{−}, a molecule that is capable of inducing oxidative reactions, mediates the activation of p38 MAPK. The fact that complete inhibition of arachidonic acid release was achieved with the MEK1/2 inhibitor U0126 suggests that the p38 MAPK pathway is initiated by metabolites downstream of MEK1/2. This finding is supported by the fact that ONOO\textsuperscript{−}-induced p38 phosphorylation was inhibited by U0126 (Fig. 7A). Here, it was also demonstrated that the addition of arachidonic acid activates p38 in smooth muscle cells, a process that is inde-
ependent of the MEK/ERK pathway (Fig. 7B). Recently, it was shown that cPLA2-dependent arachidonic acid metabolites contribute to norepinephrine-induced activation of p38 MAPK in vascular smooth muscle cells (30). In particular, the arachidonic acid metabolites of lipoygenase, 5(S)-hydroxyeicosatetraenoic acid (HETE), 12(S)-HETE, and 15(S)-HETE, are known to activate p38 MAPK (30, 51, 67). The PGHS metabolite, PGE2, is also known to cause p38 MAPK activation (18). Thus formation of arachidonic acid metabolites may cause a positive feedback mechanism in the cPLA2-catalyzed arachidonic acid process. Taken in total, our findings indicate that the major ONOO−-driven pathway in smooth muscle cells leading to arachidonic acid release and subsequent eicosanoid production is the MEK/ERK pathway and that initiation of the p38 MAPK pathway is dependent on metabolites generated from the MEK/ERK pathway.

Involvement of CaMKII in ONOO−-mediated activation of ERK1/2 was previously reported in PC-12 cells (29). Here, we demonstrate that CaMKII is also activated by ONOO− in rat smooth muscle cells (Fig. 5). CaMKII requires Ca2+ and calmodulin (CaM) for activation, and, once activated, CaMKII autophosphorylates at Thr286, which allows phosphorylation of target substrates (17, 61). Phosphorylation of Thr286 results in partial activation of the kinase, even in the absence of Ca2+/CaM (61). The CaMKII-specific inhibitor KN-93 (40) can prevent CaMKII autophosphorylation. Although norepinephrine-induced activation of CaMKII leads to cPLA2 phosphorylation on Ser515 (but not on Ser505 or Ser727) with concomitant arachidonic acid release (44), this was not observed in our studies using ONOO− as an agonist. Our studies indicate that full-length cPLA2 is activated with phosphorylation occurring at Ser505.

In addition to phosphorylation, cPLA2 activation also requires a source of Ca2+ (25, 37). In this regard, ONOO− exposure is known to cause increased cytosolic Ca2+, which depends on the presence of extracellular Ca2+ (3). Coupled with the role of ONOO− in cellular signaling, we thus examined the role of Ca2+ as a necessary requirement for the activation of cPLA2. Ca2+ chelation was found to prevent the release of arachidonic acid after ONOO− addition (Fig. 8). These results implicate a role for the calcium-dependent form of PLAs, which requires Ca2+ for the translocation of cPLA2 from the cytosol to membranes where phospholipids are present (70). It could be argued that the increased permeability of Ca2+ is due to membrane damage by ONOO−. However, the fact that maximal cPLA2 activation and prostaglandin production occurred 20–30 min after ONOO− addition and the fact that this process was prevented by specific inhibitors of cell signaling molecules indicate that cell damage is unlikely to be the cause.

Finally, the role of ONOO− in cellular signaling has implications in the context of inflammation and, in particular, vascular disease. Atherosclerotic lesions have been shown to contain iNOS, which is capable of generating both NO- and superoxide (69) and may promote the activity of ONOO− (8). The rate of ONOO− production is three times faster than the rate by which superoxide is scavenged by superoxide dismutase (4), indicating that ONOO− formation is favorable in a pathophysiological setting. Prolonged exposure to ONOO− leads to Tyr nitration that will inhibit PGI2 synthase (59, 73–75) and PGHS (13), thus limiting the production of PGI2 (64). Indeed, nitrated PGHS-1 has been observed in human atherosclerotic lesions, although the mechanism of its formation remains unknown (13). Although the direct effects of ONOO− in the pathology of vascular disease remain a question, it is apparent that ONOO− mediates the production of eicosanoids via the activation of ERK in vascular smooth muscle cells. In this regard, it is interesting to note that elevated levels of activated ERK1/2 have been found in rabbit atherosclerotic lesions compared with control tissue (26). It is possible that the localization of iNOS in the vicinity of an atherosclerotic lesion elevates ONOO− levels, thereby activating the ERK family of MAPKs. Thus, although the actions of ONOO− are considered deleterious on an acute chemical basis, ONOO− may provide the cell with a means to combat some of the clinical manifestations of inflammation through specific eicosanoids before Tyr nitration becomes prominent. Increased PGHS-2 expression in atherosclerosis (60) can contribute to the increased eicosanoid production that is observed in atherosclerotic patients (6, 19). However, the fact that iNOS gene removal causes a reduction in PGE2 synthesis in mice (42) argues for a role of RNS in this process. It is intriguing to consider that ONOO−-induced signaling pathways (in addition to ONOO− activation of PGHS) may be contributing to increased eicosanoid output in atherosclerotic patients. Our results indicate that studies investigating the role of the NO-pathway on eicosanoid production need to consider the contribution of signaling pathways initiated by RNS. These findings may provide evidence for a new role of ONOO− during vascular disease.

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REFERENCES

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