Role of Hydroperoxyeicosatetraenoic Acids in Oxidative Stress-induced Activating Protein 1 (AP-1) Activity*

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We have previously reported that hydrogen peroxide, an active oxygen species and a cellular oxidant, induces c-Fos and c-Jun mRNA expression and DNA synthesis in vascular smooth muscle cells and that these events require arachidonic acid release and metabolism through the lipoxygenase pathway. Here we have identified the eicosanoids that mediate the hydrogen peroxide-induced growth-related events in these cells. Hydrogen peroxide stimulated the production of 12- and 15-hydroperoxyeicosatetraenoic acids in vascular smooth muscle cells. Both 12- and 15-hydroperoxyeicosatetraenoic acids induced the expression of c-Fos and c-Jun protein and increased activating protein 1 (AP-1) activity, as measured by AP-1-DNA binding and AP-1-dependent human collagenase promoter-driven chloramphenicol acetyltransferase reporter gene transcription. Hydrogen peroxide and arachidonic acid also induced the expression of c-Fos and c-Jun protein and AP-1 activity. Nordihydroguaiaretic acid, an inhibitor of the lipoxygenase pathway, significantly inhibited both hydrogen peroxide and arachidonic acid-stimulated c-Fos and c-Jun protein expression and AP-1 activity. Together, these findings suggest that hydrogen peroxide induces the production of eicosanoids and that the eicosanoids are potential mediators of the oxidative stress-stimulated growth-related events in vascular smooth muscle cells.

Active oxygen (AO)† species such as superoxide anion, hydrogen peroxide, and hydroxyl radicals are constantly formed in all aerobic cells as a result of mitochondrial electron transport and several enzymatic reactions such as xanthine oxidase, NADH/NADPH oxidase, monoxygenases, and cyclooxygenases (1–3). These AO species damage DNA, lipids, and protein (1–5). However, several cellular antioxidant defenses such as superoxide dismutase, catalase, glutathione peroxidase, vitamin C, and vitamin E protect cells from such toxins (1–3). The production of AO species in any given cell varies, depending on its state of metabolic activity or stimulus. For example, neutrophils generate a large amount of AO species upon activation (4). Likewise, ischemic reperfusion results in increased production of AO species (5). In addition, some AO species such as hydrogen peroxide penetrate the plasma membrane (5). Therefore, the generation of AO species in one cell type can affect levels in neighboring cells or cell types. AO species levels may also increase in cells when the ability of the cells to scavenge them decreases. All of these conditions with deficient protective mechanisms may lead cells to oxidative stress.

Oxidative stress has been implicated in the pathogenesis of diseases such as atherosclerosis and cancer, as well as in aging and in some inflammatory disorders (5–9). The major effects of AO species are damage to DNA and oxidative inactivation of certain enzymes (1, 2). However, recent work from several laboratories shows that AO species under nontoxic levels can stimulate the expression of early growth-response gene mRNAs and can cause growth in some cell types (10–14). In studying the role of AO species in atherogenesis, we observed that AO species stimulate c-Fos and c-Jun mRNA expression and DNA synthesis in VSMC (15). We demonstrated that such AO species-induced growth-related events require arachidonic acid release and metabolism through the lipoxygenase pathway (16–18). In this communication, we report identification of arachidonic acid metabolites that mediate oxidative stress-induced growth-related events in VSMC. Specifically, hydrogen peroxide-stimulated production of 12- and 15-HETEs in VSMC and these eicosanoids were found to induce c-Fos and c-Jun expression and AP-1 activity.

EXPERIMENTAL PROCEDURES

Materials—Arachidonic acid, 12- and 15-hydroperoxyeicosatetraenoic acids, and prostaglandin E2 were from Cayman Chemical Co., Inc. (Ann Arbor, MI). Anti-c-Fos and anti-c-Jun rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Oncogene Science Inc. (Uniondale, NY), respectively. Nordihydroguaiaretic acid was purchased from Aldrich. AP-1 and NF-kB consensus double-stranded oligonucleotides and T4 polynucleotide kinase were from Promega (Madison, WI). [5,6,8,9,11,12,14,15-3H]Arachidonic acid (221 Ci/mmol), [γ-32P]ATP (3000 Ci/mmol), and [14C]chloramphenicol (59 mCi/mmol) were obtained from Dupont NEN.

Cell Culture—VSMC were isolated from the thoracic aortas of 200–250-g male Sprague-Dawley rats by enzymatic digestion as described earlier (15). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified 5% CO2 atmosphere.

Extraction of [3H]Arachidonic Acid Metabolites—VSMC were plated onto 100-mm dishes and grown in DMEM containing 10% calf serum and 15 μCi/ml [3H]arachidonic acid (10 μM final concentration) for 24 h. Cells were then growth-arrested by incubation in DMEM containing 0.1% calf serum and 5 μCi/ml [3H]arachidonic acid for 72 h. In all experiments, cells were used that incorporated approximately the same amounts of the label as assessed by counts/min/mg of protein. After the growth arrest period, the medium was removed, and cells were washed twice with phosphate-buffered saline (PBS) and stimulated with and without hydrogen peroxide (200 μM) in DMEM containing 0.1% calf
serum. Radiolabeled arachidonic acid metabolites released into the medium were extracted and analyzed as described previously (19). Western Blot Analysis—VSMC were plated onto 60-mm dishes and grown in DMEM containing 10% calf serum. At 70–80% confluence, cells were growth-arrested by incubating for 72 h in DMEM containing 0.1% calf serum. Growth-arrested VSMC were treated with and without the agent of interest for the indicated time periods at 37 °C. After treatments, the medium was removed, and cells were rinsed with cold PBS; cells were frozen immediately in liquid nitrogen. 200 μl of lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, and 1 mM sodium orthovanadate) was added to the frozen monolayers, thawed on ice for 15 min, and scraped into 1.5-ml Eppendorf tubes. The cell lysates were cleared by centrifugation at 12,000 × g for 20 min at 4 °C. The protein concentration of the supernatants was determined using the Bradford reagent (Bio-Rad). Cell lysates containing equal amounts of protein (30 μg/lane) were analyzed by Western blotting for c-Fos and c-Jun proteins using specific antibodies as described earlier (20).

Nuclear Extract Preparation and Gel Mobility Shift Assays—Growth-arrested VSMC were treated with and without the agents of interest for the indicated times, and nuclear extracts were prepared as described earlier (20). Protein-DNA complexes were formed by incubating 5 μg of nuclear protein in a total volume of 20 μl consisting of 15 μl Hesep, pH 7.9, 3 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 4.5 μg of bovine serum albumin, 2 μg of poly(dI-dC), 15% glycerol, and 100,000 cpm of 32P-labeled oligonucleotide probe for 20 min at 30 °C. Protein-DNA complexes were resolved on a 4% polyacrylamide gel using 0.25 × TBE buffer (1 × TBE = 50 mM Tris borate, pH 8.3, and 1 mM EDTA). Double-stranded oligonucleotides (AP-1, 5′-CGCTTGATGAGTCAGC-3′; NF-κB, 5′-AGTTGACCTTTCCAGGCAGC-3′) were labeled with γ-32P-ATP using a T4 polynucleotide kinase kit per the protocol of the supplier (Promega). Unincorporated nucleotides were removed by chromatography in a G-25 spin column (Bio-Rad).

Transient Transfection and CAT Assays—Existing VSMC culture was split evenly into 100-mm dishes and grown in DMEM containing 10% calf serum overnight. Cells were transfected with the plasmid DNA of interest (25 μg/100-mm dish) using a calcium phosphate precipitation method as described by Angel et al. (21). 16 h after initiation of transfection, cells were washed with PBS and quiesced by incubating in DMEM containing 0.1% calf serum for 5 min at 37 °C and then stimulated with an appropriate agent for 6 h. Cells were again washed with PBS, scraped in 1 ml of TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 150 mM NaCl) into an Eppendorf tube, and pelleted by centrifugation at 12,000 × g for 1 min at 4 °C. The cell pellet was suspended in 100 μl of cold 0.25% Triton X-100 (Tris-HCl buffer, pH 7.5, and the cells were lysed by three repeated freeze-thaw cycles. Cell debris was removed by centrifugation at 12,000 × g for 5 min at 4 °C. Protein concentration of the supernatant was determined as described above. CAT activity was measured by the method of Gorman et al. (22). In brief, 50 μg of protein from each condition was incubated with 20 μl of 4 mM acetyl-CoA, 32.5 μl of 1% Triton X-100, pH 7.5, 4 μl of 50 μCi/ml [3H]chloramphenicol in a total volume of 150 μl at 37 °C for 20 min. Controls without cell extract and/or with non-transfected cell extracts were incubated simultaneously. Acetylated and non-acetylated chloramphenicol were extracted with ethyl acetate and separated by thin layer chromatography on Silica Gel 1B plates using a chloroform: methanol mixture (19:1) as solvent. Following autoradiography, percent acetylation was calculated by cutting out the corresponding acetylated and non-acetylated spots of the TLC plate and counting the radioactivity as described above.

RESULTS AND DISCUSSION

To determine the arachidonic acid metabolites whose synthesis was modulated in response to oxidative stress, VSMC were labeled with [3H]arachidonic acid, growth-arrested, and treated with and without hydrogen peroxide (200 μM) for 1 h. The labeled arachidonic acid metabolites released into the medium were extracted and analyzed by reverse phase HPLC. Constitutive but low levels of 15-HETE were released from [3H]arachidonic acid-prelabeled VSMC (Fig. 1A). Hydrogen peroxide treatment increased production of 12- and 15-

FIG. 1. Hydrogen peroxide induces synthesis of eicosanoids in VSMC. [3H]Arachidonic acid-labeled and growth-arrested VSMC were stimulated for 1 h with and without 200 μM hydrogen peroxide. The labeled arachidonic acid metabolites released into the culture medium were extracted and analyzed by reverse phase HPLC. Retention times were compared with 3H-labeled standards. A, labeled arachidonic acid metabolites formed by VSMC; B, labeled arachidonic acid metabolites formed by VSMC in response to hydrogen peroxide; and C, labeled arachidonic acid metabolites formed by VSMC in response to hydrogen peroxide in the presence of NDGA (10 μM), a lipoperoxidase inhibitor. Retention times of authentic 3H-labeled eicosanoid standards are indicated with arrows in panel A as follows: 1, PGE2; 2, PGF2α; 3, leukotriene C4; 4, leukotriene B4; 5, 12(S)-hydroxyeicosatetraenoic acid; 6, 15-HETE; 7, 12-HETE; 8, 5-HETE; and 9, arachidonic acid.
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hydrogen peroxide both in the presence and absence of NDGA (cells were pretreated with 10 μM NDGA, for 30 min prior to hydrogen peroxide addition), and the released arachidonic acid metabolites were measured as described above. NDGA completely blocked the hydrogen peroxide-induced production of 12- and 15-HPETE (Fig. 1C). NDGA enhanced hydrogen peroxide-induced PGE₂ synthesis. This finding is expected because inhibition of the lipoxygenase pathway results in increased arachidonic acid substrate availability for metabolism by cyclooxygenase. This result also argues strongly against the antioxidant effect of NDGA because a decrease both in HPETE and PGE₂ production induced by hydrogen peroxide would be otherwise expected. Collectively, these observations suggest that hydrogen peroxide induces the synthesis of eicosanoids in VSMC and that both the lipoxygenase and the cyclooxygenase pathways are regulated in response to oxidative stress.

Since hydrogen peroxide-induced growth-related events are sensitive to inhibition by NDGA, we wanted to test whether 12- and 15-HPETE mediate oxidative stress-induced c-Fos and c-Jun expression. To address this issue, we studied the effect of 12- and 15-HPETE on c-Fos and c-Jun expression in VSMC. Growth-arrested VSMC were treated for various time periods with 12- and/or 15-HPETE (1 μM), and cell lysates were prepared. 30 μg of protein from each time period were analyzed by Western blotting for c-Fos and c-Jun proteins using their respective antibodies. As shown in Fig. 2A, both 12- and 15-HPETE stimulated the expression of c-Fos and c-Jun proteins in a time-dependent manner. A maximum 3-fold increase in c-Fos and c-Jun protein expression in response to 12- and 15-HPETE was observed at 2 h after initiation of treatment, and this response persisted for at least 6 h. Note that 12-HPETE was found to be more potent in inducing c-Fos and c-Jun expression than was 15-HPETE. To determine whether hydrogen peroxide, which caused an increase in eicosanoid production in VSMC, and arachidonic acid, the precursor of eicosanoids, also stimulate c-Fos and c-Jun expression, growth-arrested VSMC were treated with these agents for 2 h, and cell lysates were prepared. Cell lysates containing equal amounts of protein (30 μg) from each condition were then analyzed for c-Fos and c-Jun proteins by Western blotting as described above. Both hydrogen peroxide (200 μM) and arachidonic acid (20 μM) treatments caused significant increases in the expression of c-Fos and c-Jun as compared with their controls, and these responses were inhibited by NDGA (Fig. 2B).

As c-Fos and c-Jun are the major constituents of the transcriptional factor AP-1 (23, 24) and HPETEs increased the expression of these protooncogenes, we tested the effect of the same eicosanoids on AP-1 activation in VSMC. AP-1 activity was measured by its ability to bind to the 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE), TGACTCA. Nuclear extracts of 12-HPETE-treated VSMC displayed 2–3-fold more TRE binding activity than extracts of untreated VSMC, as determined by gel mobility shift assay (Fig. 3A). Almost maximal increases in AP-1-TRE binding activity in response to 12-HPETE treatment were observed by 2 h, and these responses were persistent for at least 6 h. Addition of NFκB consensus cold oligonucleotide had no effect on 12-HPETE-induced AP-1-TRE binding (Fig. 3A, lane 5). On the other hand, the addition of excess cold TRE to the reaction mix reduced AP-1 binding to 125I-labeled TRE (Fig. 3A, lanes 6 and 7). The latter two observations clearly indicate that the AP-1-TRE binding activity observed in the nuclear extracts of 12-HPETE-treated VSMC is specific. A time course of 15-HPETE-stimulated AP-1-TRE binding activity yielded results similar to those obtained with 12-HPETE (Fig. 3A). Addition of excess cold TRE (100-fold) to the reaction mix also competed with the labeled probe for binding to AP-1 in the nuclear extracts of 15-HPETE-treated VSMC (data not shown). As noticed in Fig. 3, differential basal AP-1-DNA binding activities were observed between different experiments. This may be due to different levels of VSMC growth arrest in different experiments. Nuclear extracts of VSMC that were treated with hydrogen peroxide and/or arachidonic acid for 2 or 4 h had significantly higher AP-1-TRE binding activities in comparison with extracts of untreated cells (Fig. 3B). NDGA, which alone had no effect, significantly inhibited (75%) hydrogen peroxide and arachidonic acid-induced AP-1 activity (Fig. 3B). Cell extracts of VSMC that were transiently transfected with −73/+63 collagenase-CAT and exposed to hydrogen peroxide (100 μM), arachidonic acid (20 μM), 12-HPETE (1 μM), or 15-HPETE (1 μM) for 6 h also showed a 2-fold increase in CAT activity as compared with extracts of untreated cells (Fig. 4). The -fold increases in CAT activities in treated versus control cells are comparatively less than the apparent increases in AP-1-TRE binding assays. This may be explained by differences between episomal and chromosomal gene regulation.

The novel finding of the present study is that hydrogen peroxide induces the synthesis of 12- and 15-HPETE in VSMC and that these eicosanoids possess the ability to activate AP-1. Work from several laboratories suggests that lipoxygenase inhibitors exhibit antiproliferative activity (25, 26). In addition, the ability of arachidonic acid and its lipoxygenase metabolites such as 12- and 15-HPETE to act as mitogens of several cell types has been demonstrated (27, 28). Some studies have re-
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FIG. 3. 12- and 15-HPETE, hydrogen peroxide, and arachidonic acid stimulate AP-1-DNA binding activity in VSMC. A, nuclear extracts (5 μg) of 0 (control), 2, 4, and 6 h of 12-HPETE-treated VSMC (lanes 1–4, respectively) were incubated with radiolabeled AP-1-specific oligonucleotide, and the AP-1-DNA complex was separated from the free probe on a 4% non-denaturing polyacrylamide gel. Cold NF-κB (100-fold, lane 5) and AP-1 (10-fold, lane 6; and 100-fold, lane 7), respectively, were added to the nuclear extract of 12-HPETE-treated (6 h) VSMC prior to incubation with radiolabeled AP-1 oligonucleotide probes. Lanes 8–12 are nuclear extracts of 0 (control), 1, 2, 4, and 6 h of 15-HPETE-treated VSMC, respectively, that were incubated with a radiolabeled AP-1 oligonucleotide probe. B, treatments of VSMC are as follows: lane 1, control; lane 2, hydrogen peroxide for 2 h; lane 3, hydrogen peroxide for 4 h; lane 4, arachidonic acid for 2 h; lane 5, arachidonic acid for 4 h; lane 6, hydrogen peroxide for 2 h but in the presence of NDGA; lane 7, arachidonic acid for 2 h but in the presence of NDGA; and lane 8, NDGA alone. 5 μg of nuclear proteins from each treatment were incubated with radiolabeled AP-1 oligonucleotide probe, and the products were separated on PAGE as described above. NDGA, wherever used, was added 30 min before the addition of the agent.

ported that growth factors such as epidermal growth factor and serum require arachidonic acid release and metabolism through the lipoxygenase pathway for induction of growth in certain cell types (29, 30). Natarajan et al. (31) have demonstrated that angiotensin II requires 12-HPETE production for its hypertrophic effect in rabbit aortic smooth muscle cells. We have previously shown that oxidative stress induces c-Fos and c-Jun mRNA expression and DNA synthesis in VSMC and that these events require arachidonic acid release and metabolism via the lipoxygenase pathway (15–17). Because the lipoxygenase metabolites of arachidonic acid have been shown to be critical in the modulation of growth in response to certain peptide growth factors and hydrogen peroxide increased the production of lipoxygenase metabolites of arachidonic acid such as 12- and 15-HPETE, it is likely that these eicosanoids, at least in part, mediate the oxidative stress-induced growth-related events in VSMC. This idea can be further supported by our finding that 12- and 15-HPETE are capable of stimulating c-Fos and c-Jun expression and AP-1 activity in VSMC. However, a role for other enzymatic or non-enzymatic products of arachidonic acid or linoleic acid in oxidant-mediated cell responses cannot be ruled out. In fact, since hydrogen peroxide treatment, in addition to 12- and 15-HPETE, increased the production of at least one more [3H]arachidonic acid-derived product that elutes between 76 and 78 min in this reverse phase HPLC system and the synthesis of this compound was somewhat sensitive to inhibition by NDGA, it is possible that this molecule may also be involved in oxidant-mediated cell responses. Future studies should identify this compound and examine whether it modulates growth response events in these cells. In addition, we recently found that 4-hydroxynonenal, an aldehyde that can be produced non-enzymatically from arachidonic acid, linoleic acid, or their hydroperoxides (32), stimulates c-Fos and c-Jun expression and DNA synthesis in VSMC. Thus, polysaturated fatty acids, their hydroperoxides, and probably the non-enzymatic breakdown products of these lipid molecules may alone or in combination play an important role in the pathogenesis of lesions such as atherosclerosis and cancer that are linked to oxidative stress. Indeed, increased levels of HPETEs were reportedly observed in atherosclerotic arteries (33, 34) and in certain forms of cancer (35), and the enzymes responsible for their production are present in many cell types including arterial smooth muscle cells (31, 36, 37). Since hydrogen peroxide was capable of up-regulating the levels of HPETEs and these HPETEs are growth modulators to some cells, one possible mechanism by which oxidative stress could influence atherosclerosis is by stimulation of smooth muscle cell growth via generation of eicosanoids such as the ones we observed.

Smooth muscle cell migration to and their multiplication in intima appeared to be one of several determinant factors in atherosclerosis (38). HPETEs were reported to be potent chemotactants for various cell types including VSMC (39). Since hydrogen peroxide increased the production of 12- and 15-HPETE, it is also tempting to speculate that oxidative stress modulates VSMC migration in an autocrine manner. This event can even be further compounded by the fact that VSMC production of HPETEs induced by oxidative stress may influence migration of neutrophils, macrophages, and platelets

G. N. Rao, J. Ruef, and M. S. Runge, unpublished observations.
to and their deposition in the intima. If this is, indeed, true then antioxidant therapies as suggested by Steinberg (40) could modify the atherosclerosis process.

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