Induction of Rat Aortic Smooth Muscle Cell Growth by the Lipid Peroxidation Product 4-Hydroxy-2-Nonenal

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Background—Atherosclerotic lesion formation is a complex process, in part mediated by inflammatory and oxidative mechanisms including lipid peroxidation. To further characterize the potential role of lipid peroxidation products in atherogenesis, we studied the effects of 4-hydroxy-2-nonenal (HNE) on rat aortic smooth muscle cell growth.

Methods and Results—HNE, at concentrations of 1.0 and 2.5 μmol/L, significantly stimulated rat aortic smooth muscle cell growth as determined by cell counts, [3H]-thymidine uptake, and incorporation of bromo-deoxyuridine. To characterize the mechanism of HNE-induced mitogenesis, its effect on activation of intracellular growth signaling pathways was examined. Treatment with HNE resulted in activation of extracellular signal-regulated protein kinases ERK1 and ERK2, induction of c-fos and c-jun protein expression, and an increase in transcription factor AP-1 DNA binding activity. In addition, HNE induced expression of platelet-derived growth factor-AA (PDGF-AA) protein, and an anti–PDGF-AA antibody specifically inhibited HNE-mediated DNA synthesis, suggesting that growth factor induction may play a role in HNE-induced vascular smooth muscle cell growth. The role of redox-sensitive mechanisms in this process was further supported by the observation that HNE-induced DNA synthesis and AP-1 activation were inhibited by the antioxidants N-acetylcysteine and pyrrolidine dithiocarbamate.

Conclusions—These data demonstrate that HNE, one of several important lipid peroxidation products, induces rat aortic smooth muscle cell growth through redox-sensitive mechanisms and growth factor expression. These observations are consistent with a role for lipid peroxidation products in vascular smooth muscle cell growth in atherogenesis. (Circulation. 1998;97:1071-1078.)

Key Words: atherosclerosis † oxidation † lipids † signal transduction † mitogens

Atherosclerosis is a multifactorial disease that ultimately results in thickening of the arterial wall with a concomitant decrease in lumen diameter. Two mutually compatible theories have been proposed to explain the development of atherosclerotic lesions. The “oxidation” hypothesis of atherogenesis emphasizes oxidative modification of lipoproteins leading to recruitment of macrophages and other inflammatory cells into lesions as the key event in atherogenesis.1,2 The “response-to-injury” hypothesis posits VSMC proliferation as a hallmark of the formation and progression of atherosclerotic lesions.3

Although a large body of data supports a role for both oxidative modification of lipids and VSMC mitogenesis in atherogenesis, mechanistic links between these two events have only recently been proposed. After an insult to the vessel wall, inflammatory mechanisms of protection and repair are initiated, an important component of which is the generation of reactive oxygen species (ROS). Activated leukocytes produce superoxide anion, hydrogen peroxide, and hydroxyl radicals through activation of NADH/NADPH oxidases.4,5 In addition, endothelial cells and VSMC are a source of significant free radical production.6 These locally generated ROS result in oxidation of both circulating and membrane-bound lipids, perhaps accounting for the increased levels of lipid peroxidation products reported in patients with atherosclerosis.7 It has also been demonstrated that ROS and oxidized lipids both stimulate VSMC growth.8-12 Furthermore, PDGF, which has been implicated in VSMC proliferation in atherosclerosis, requires ROS generation for its mitogenic effects.13 Finally, antioxidants such as probucol, β-carotene and α-tocopherol inhibit vascular lesion formation in hyperlipidemic animals14-16 and thiol antioxidants, such as PDTC, inhibit vascular cell growth in vitro.17 Together, these data support a mechanistic relationship between oxidant generation, lipid peroxidation, VSMC growth, and atherogenesis.18,19

In the present study, we hypothesized that HNE, a component of oxidatively modified lipids, might link oxidative events to VSMC proliferation in atherogenesis. HNE is a major product of lipid peroxidation that is produced by β-scission of alkoxyl radicals in polyunsaturated fatty acids such as arachi-
HNE is detectable in the plasma of healthy probands (up to 1.4 µmol/L) and is present in tissues at concentrations of up to 20 µmol/L. Several observations support the hypothesis that HNE may provide a link between oxidant generation, lipid peroxidation, and VSMC proliferation in atherogenesis. First, HNE is a component of oxidized LDL and is found in atherosclerotic lesions. Immunoreactive HNE is present at all stages of human atherosclerosis but not in normal human arteries and has also been identified in the neointima of balloon-injured baboon arteries. Second, lipid peroxidation products, and specifically HNE, stimulate chemotaxis and growth in other systems and have been implicated in other pathological conditions thought to be related to oxidative stress. In these settings, it has been proposed that HNE and related aldehydes act to amplify the cellular effects of their free radical precursors.

Here we report that HNE stimulates proliferation of RASM. This dose-dependent proliferation is associated with induction of mitogenic signaling events in RASM, including activation of ERKs, increases in c-fos and c-jun protein expression, and enhanced AP-1 DNA binding activity. HNE induces PDGF-AA protein expression and an anti-PDGF-AA antibody ameliorates the effects of HNE on RASM growth, suggesting that growth factor induction may be an important intermediary step in HNE-induced vascular smooth muscle cell growth. Finally, the effects of HNE on proliferation and mitogenic signaling are inhibited by the antioxidants NAC and PDTC, and HNE rapidly downregulates cellular thiol groups, suggesting that the effects of HNE are at least partly mediated through redox-sensitive events.

Methods

Materials

Unless otherwise mentioned, all chemicals were purchased from Sigma Chemical Co. HNE was obtained from Cayman Chemical Co. (methyl-[^2]H)-thymidine was obtained from DuPont NEN and [γ-[^32]P]ATP from Amersham Co. Antibodies for c-fos, c-jun, PDGF-AA, FLK-1, and rabbit IgG (PE) were from Santa Cruz Biotechnology Inc., Oncogene Science, Genzyme, and Boehringer Mannheim, respectively. Double-stranded oligonucleotides containing a consensus AP-1 recognition sequence were purchased from Promega.

Cell Culture

RASM were isolated from the thoracic aortas of 200- to 250-g male Sprague-Dawley rats by enzymatic digestion and kept in culture as described previously. For most experiments, cells at 80% confluency were made quiescent by incubation for 72 hours in DME containing 0.1% fetal bovine serum. For cell number and DNA synthesis experiments, cells were grown to 50% confluency. RASM were used at passages 6 to 15, since no differences in responsiveness were noted within this range. Low levels of serum were maintained during quiescence to prevent slow apoptosis that accompanies complete serum deprivation in vascular smooth muscle cells.

Cell Number

Cells were growth-arrested in 60-mm dishes, as described above, and HNE or serum was added to the cells. After 72 hours the cells were trypsinized, washed in PBS, and counted in a Coulter counter (Coulter Electronics).

DNA Synthesis

Growth-arrested RASM were treated with HNE or 10% serum for 48 hours.[^22] A 442[^23] HNE was detected in the plasma of healthy probands, and it is found in atherosclerotic lesions.[^24] Immunoactive HNE is present at all stages of human atherosclerosis but not in normal human arteries and has also been identified in the neointima of balloon-injured baboon arteries.[^25] Second, lipid peroxidation products, and specifically HNE, stimulate chemotaxis and growth in other systems and have been implicated in other pathological conditions thought to be related to oxidative stress. In these settings, it has been proposed that HNE and related aldehydes act to amplify the cellular effects of their free radical precursors. Here we report that HNE stimulates proliferation of RASM. This dose-dependent proliferation is associated with induction of mitogenic signaling events in RASM, including activation of ERKs, increases in c-fos and c-jun protein expression, and enhanced AP-1 DNA binding activity. HNE induces PDGF-AA protein expression and an anti-PDGF-AA antibody ameliorates the effects of HNE on RASM growth, suggesting that growth factor induction may be an important intermediary step in HNE-induced vascular smooth muscle cell growth. Finally, the effects of HNE on proliferation and mitogenic signaling are inhibited by the antioxidants NAC and PDTC, and HNE rapidly downregulates cellular thiol groups, suggesting that the effects of HNE are at least partly mediated through redox-sensitive events.

Selected Abbreviations and Acronyms

BrdU = bromo-deoxyuridine
BSO = L-buthionine sulfoximine
ERKs = extracellular signal-regulated protein kinase
HNE = 4-hydroxy-2-nonenal
NAC = N-acetylcysteine
PDGF = platelet-derived growth factor
PDTC = prlolidine diithiocarbamate
RASM = rat aortic smooth muscle cell(s)
VSMC = vascular smooth muscle cell(s)
Figure 1. HNE increases RASM number and DNA synthesis. A, Growth-arrested RASM were treated with and without HNE in the doses indicated or 10% serum for 3 days and cells were counted using a Coulter counter. Results are shown as mean ± SEM (n = 9). B, Growth-arrested RASM were treated with and without HNE or serum in the doses indicated in the presence or absence of NAC (20 mmol/L) for 48 hours. Cells were exposed to 1 μCi/mL [3H]-thymidine for the last 24 hours in the 48 hour incubation period and trichloroacetic acid-precipitable material was measured as a marker of DNA synthesis. Results are shown as mean ± SEM (n = 6). C, To obtain additional quantification of DNA synthesis, a sensitive immunoassay based on BrdU incorporation was performed. Growth-arrested RASM were treated with or without HNE or serum for 24 hours in the presence of 10 μmol/L BrdU. Incorporation of BrdU was analyzed by ELISA. Results are expressed as mean ± SEM of 9 replicates. *P < 0.05 compared with control.

arrested RASM to HNE (1.0 to 5.0 μmol/L), cell number was determined after 3 days of treatment. Cell number was significantly increased after treatment with either 1.0 μmol/L or 2.5 μmol/L HNE (by 41% and 37%, respectively, P < 0.05) compared with controls (Fig 1A). These effects of HNE on cell number were similar in magnitude to the mitogenic effects of other growth factors that have been studied, but less than that of 10% serum. Interestingly, at a higher concentration (5.0 μmol/L), HNE treatment resulted in a decrease in cell number by 34% compared with control (P < 0.05). This may have been due to the known toxicity of HNE when used at this concentration for extended time periods. HNE treatment of RASM induced a similar effect on DNA synthesis as determined by measurement of [3H]-thymidine incorporation. Treatment with HNE at 1.0 μmol/L and 2.5 μmol/L over 48 hours increased [3H]-thymidine incorporation by 41% and 30%, respectively (P < 0.05, Fig 1B). As with cell growth, treatment of RASM with a higher dose of HNE (5.0 μmol/L) resulted in decreased DNA synthesis. To confirm these results, we also measured the effect of HNE on DNA synthesis by determining incorporation of BrdU in RASM, both in vitro (Fig 1C) and ex vivo in cultured rat aorta sections (data not shown). As shown in Fig 1C, treatment of RASM with HNE at 1.0 μmol/L and 2.5 μmol/L over 24 hours resulted in increased incorporation of BrdU (110% and 82%, respectively, P < 0.05) compared with controls. Additional experiments were performed to determine whether pulse-labeling with [3H]-thymidine and quiescence of RASM in 0% serum would maximize the mitogenic effect of HNE. Although a slightly more marked effect was observed under these conditions (see Fig 6), prolonged lack of serum can lead to apoptosis in RASM and other VSMCs, potentially confounding the effects of higher doses of HNE. For this reason, the majority of the experiments reported here were performed using the standard conditions described in the “Methods” section. Together these data indicate that HNE induces RASM growth.

HNE Activates Early Mitogenic Signaling Events in RASM

The ERKs family of mitogen-activated protein kinases is a major pathway by which information from extracellular signaling events is transduced to the nucleus. Because ERKs are implicated in mitogenic responses in VSMC induced by ROS such as H2O2 and O2−, we examined whether HNE also activates the ERKs signaling pathway. ERKs activities were measured in HNE-treated and untreated RASM with an in-gel kinase assay, with myelin basic protein as a substrate. In this assay, after fractionation of cell lysates by SDS-PAGE and renaturation, ERKs activity is determined based on phosphorylation of myelin basic protein contained in the gel. HNE (2.5 μmol/L) rapidly and transiently activated both ERK1 and ERK2 (Fig 2). Maximal activation of both ERK1 and ERK2 (fourfold as determined by densitometry) occurred within 5 minutes. By 40 minutes, the activities of these enzymes had returned to basal levels. This time course is similar to that shown in VSMC for O2− and angiotensin II and more rapid than that for hydrogen peroxide. These data indicate that at least one effect of HNE is to rapidly activate critical mitogenic signaling pathways in RASM.

HNE Induces Mitogenic Nuclear Events in RASM

The observation that HNE activates ERK1 and ERK2 raised the possibility that the mitogenic effect of HNE is ultimately mediated by activation of the nuclear factor AP-1. AP-1 activation is a well-defined mechanism by which numerous mitogens that signal through ERK1 and ERK2 stimulate...
VSMC growth.\textsuperscript{5,44} AP-1 activation has also been implicated in ROS-mediated gene regulation.\textsuperscript{45} To address this hypothesis, we first determined by Western blot analysis whether HNE stimulates c-jun and c-fos protein expression, because new protein expression could promote AP-1 activation. Two and 4 hours after treatment with HNE (2.5 \mu\text{mol/L}), c-jun and c-fos proteins were fourfold greater in treated than in untreated control cells (Fig 3A). Analogous to its effects on RASM growth, HNE stimulated c-fos and c-jun expression at low concentrations, but at higher concentrations decreased expression (Fig 3B).

To determine whether these HNE-induced increases in c-fos and c-jun protein translated into increased AP-1-DNA binding activity in RASM, we performed electrophoretic mobility shift analysis, using nuclear extracts from cells either treated or not treated with HNE and a \textsuperscript{32}P-labeled AP-1 consensus oligonucleotide. Nuclear proteins were isolated from growth-arrested RASM after treatment with HNE (Fig 4). HNE increased AP-1-DNA binding activity in a time-dependent manner, with maximum binding activity at 4 hours.

The Mitogenic Effect of HNE Is at Least Partly Mediated by PDGF
In addition to directly activating mitogenic signaling pathways, ROS may induce cell growth by stimulating the production and/or secretion of growth factors. In this setting, the growth factor could potentially amplify the mitogenic effect of ROS through an autocrine mechanism. PDGF-AA has been shown to mediate such autocrine events in RASM in response to xanthine/xanthine oxidase metabolism.\textsuperscript{46} To determine whether similar events occur in response to HNE, cell lysates of HNE-treated RASM were immunoblotted with an antibody specific for PDGF-AA. As shown in Fig 5, treatment with HNE (0.1 to 1.0 \mu\text{mol/L}) for 3 hours resulted in increased PDGF-AA levels. This effect was inhibited by NAC, suggesting that HNE-induced PDGF-AA synthesis is oxidant-mediated. To determine the importance of HNE-induced PDGF-AA synthesis for HNE-stimulated RASM mitogenesis, DNA synthesis was measured in the presence or absence of an anti-PDGF-AA antibody (PDGF-AA Ab). Inhibition of PDGF-AA with PDGF-AA Ab partially blocked the increase in DNA synthesis induced by HNE (60\% inhibition, \(P<.05\), Fig 6).

HNE-Induced Mitogenesis Involves a Redox-Sensitive Mechanism
The observation that the effect of HNE on thymidine uptake in RASM was blocked by treatment with the thiol antioxidant NAC (20 \mu\text{mol/L}), suggested that the effects of HNE might be mediated through redox-sensitive mechanisms (Fig 1B). To further investigate this possibility, we examined the effect of two different antioxidants on AP-1 activation by electrophoretic mobility shift assay. Both NAC (20 \mu\text{mol/L}) and PDTC (100 \mu\text{mol/L}) inhibited the HNE-induced increase in AP-1-DNA binding activity (Fig 7). Diamide was used as a positive control because it is known to induce AP-1 activation.\textsuperscript{47} Similar inhibitory effects of NAC on HNE-induced c-fos and c-jun protein expression were also observed (data not shown). On the basis of these results, it is likely that the effects of HNE on mitogenic signaling in RASM are at least in part due to redox-sensitive mechanisms.

To determine whether this potential redox-sensitive mechanism was dependent on reduction or oxidation of cellular...
thiols, we also characterized the effects of HNE on total cellular thiol content. Thiol content in HNE-treated and untreated RASM was determined using Ellman’s reagent. For comparison, thiol content was also measured in RASM treated with BSO, a glutathione synthase inhibitor that depletes cellular glutathione (Fig 8). Treatment of quiesced RASM with 1.0 μmol/L HNE for 60 minutes resulted in a 52% decrease in the cellular thiol content in comparison to untreated cells (P < .05). This effect is rapid, reaching a 26% reduction of thiol groups within 5 minutes and a 43% reduction within 15 minutes of treatment with HNE, compared with the controls (P < .05). A very similar effect was observed after treatment of RASM with 1 mmol/L BSO for 60 minutes (54% decrease in cellular thiols, P < .05). Thus HNE-mediated AP-1 activation appears to use a redox-sensitive mechanism while resulting in a decrease in total cellular thiol content. While these findings are consistent with the hypothesis that the decrease in thiol content is responsible for the effect of HNE on AP-1 activation, further investigation will be required to establish the precise molecular mechanisms involved.

Discussion

In the present study we have shown that HNE induces RASM proliferation in a dose-dependent fashion and in physiological concentrations.21,22 This effect is associated with rapid activa-
mediated by the release of growth factors. Thus it is possible that the mitogenic effect of HNE described in this report may, in part, explain the growth promoting effects of oxidized LDL on RASM proliferation. This is of interest because PDGF-AA, like other members of the PDGF family, has been previously shown to stimulate VSMC mitogenesis through autocrine production.

In this study, we demonstrate that HNE has a significant effect on key intracellular signaling events thought to be important in mitogenesis. The mitogenic sequelae of HNE support the hypothesis that metastable metabolites such as HNE function as mediators for the effects of oxygen-derived free radicals. We also demonstrated that HNE stimulates PDGF-AA expression and that this expression may account for sustained effects of HNE on RASM proliferation. This is of interest because PDGF-AA, like other members of the PDGF family, has been previously shown to stimulate VSMC mitogenesis through autocrine production.

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Lipid peroxidative reactions take place under physiological conditions in a variety of cell types, particularly in cells that are not rapidly proliferating. Of the numerous intermediates generated during lipid peroxidation, unsaturated aldehydes, such as HNE, may be particularly important in view of the high concentrations in which they are generated and their propensity to react with most cellular constituents. HNE accounts for up to 95% of the unsaturated aldehydes generated during peroxidation of ω-6-polyunsaturated fatty acids and has been shown to be highly reactive (Fig 9A). HNE can participate in a wide array of cellular reactions ranging from redox reactions and protein modification to DNA adduct formation. Because of the α, β-unsaturated structure, HNE reacts avidly with cellular nucleophiles such as thiols (Fig 9B). The thiolate anions of glutathione and cysteine add spontaneously to the electron deficient C-3 of HNE (Michael adducts). Similar Michael adducts are also formed with histidine and lysine. The formation of such adducts has been suggested to be the underlying mechanism by which HNE, generated in oxidized LDL, modifies the histidine and lysine residues of apolipoprotein B. In addition to reacting with thiols, HNE also forms Schiff’s bases with cellular amines via the functional aldehyde group.

Two independent lines of evidence implicate redox-sensitive mechanisms in the downstream growth signals activated by HNE. The first is the observation that NAC and PDTC inhibit HNE-induced mitogenic signals. Second, we demonstrated that HNE rapidly oxidizes free cellular thiol groups (Fig 8), lowering the redox state of the cell and regulating nuclear transduction. Numerous observations support the notion that HNE may provide a link between these two hypotheses. Oxidized LDL is a well-described VSMC mitogen that is intimately linked to atherogenesis. HNE is an active constituent of oxidized LDL, and antibodies raised against HNE-LDL stain histological samples of atherosclerotic lesions from animals and patients. As with HNE, at least some of the growth modulating effects of oxidized LDL are thought to be mediated by the release of growth factors. Thus it is possible that the mitogenic effect of HNE described in this report may, in part, explain the growth promoting effects of oxidized LDL reported by other investigators.
early signaling events. Multiple reactions may be involved in this process, as it has been reported that HNE-induced depletion of protein thiols occurs through thioether binding and that the reaction products, saturated aldehydes, may also undergo further rearrangements. This relates to activation of growth-related signaling in that redox-sensitive activation has been demonstrated for both the ERKs and also AP-1. Our observation that lowering intracellular thiol levels leads to activation of both ERKs and AP-1 suggests that this may be relevant in atherogenesis, at low concentrations and having cytotoxic effects at slightly higher concentrations. This may be relevant in atherogenesis, at low concentrations and having cytotoxic effects at slightly higher concentrations. Therefore, oxidants such as HNE may exert direct mitogenic effects on VSMC by activating ERKs and nuclear factors such as AP-1, the activity of which may depend on the cellular redox state.

Consistent with previous observations, HNE was found to be toxic to cells at high concentrations while lower concentrations induced cell growth and DNA synthesis (Fig 1). Similar effects have been observed with other oxidation products, such as H$_2$O$_2$ and oxidized LDL, which over a narrow concentration range can cause both proliferative and cytotoxic effects. We did not address the mechanisms by which this cytotoxicity occurred. It might represent aldehyde modification of key proteins and/or DNA necessary for cell viability. Equally plausible, however, is the idea that HNE may exhibit a narrow concentration range, stimulating cell growth at low concentrations and having cytotoxic effects at slightly higher concentrations. This may be relevant in atherogenesis, where cell growth, apoptosis, and necrosis are thought to contribute mutually to lesion formation. At 25 and 50 µmol/L HNE caused apoptosis in VSMC to an extent comparable with the effects of 50 ng/mL TNF-α (J. Ruef and M.S. Runge, unpublished data).

In summary, the data presented here are consistent with the hypothesis that oxidative stress in vascular cells establishes an amplifying circuit within which long-lived oxidants such as HNE may play a part. In such a circuit, ROS generate HNE, which results in further redox-sensitive events, leading to the expression of effector molecules such as PDGF, which create even more oxidative species as a part of their own signaling program. Such a circuit may explain how transient events such as activation of mitogen-activated protein kinases lead to further redox-sensitive events, leading to the generation of ROS at multiple points in the cascade. By virtue of their long-lived nature in comparison to intracellular oxidant sources such as hydrogen peroxide, superoxide, and hydroxyl ion, modified intermediates such as HNE may be attractive sites for intervention in such circuits.

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