4-Hydroxy-2-Nonenal Enhances Tissue Factor Activity in Human Monocytic Cells via p38 Mitogen-Activated Protein Kinase Activation-Dependent Phosphatidylinerse Exposure

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Objective—4-hydroxy-2-nonenal (HNE) is one of the major aldehydes formed during lipid peroxidation and is believed to play a role in the pathogenesis of atherosclerosis. The objective of the present study is to investigate the effect of HNE on tissue factor (TF) procoagulant activity expressed on cell surfaces.

Approach and Results—TF activity and antigen levels on intact cells were measured using factor Xa generation and TF monoclonal antibody binding assays, respectively. Exposure of phosphatidylinerse on the cell surface was analyzed using thrombin generation assay or by binding of a fluorescent dye–conjugated annexin V. 2′,7′-dichlorodihydrofluorescein diacetate was used to detect the generation of reactive oxygen species. Our data showed that HNE increased the procoagulant activity of unperturbed THP-1 cells that express traces of TF antigen, but had no effect on unperturbed endothelial cells that express no measurable TF antigen. HNE increased TF procoagulant activity but not TF antigen of both activated mononcytic and endothelial cells. HNE treatment generated reactive oxygen species, activated p38 mitogen-activated protein kinase, and increased the exposure of phosphatidylinerse at the outer leaflet in THP-1 cells. Treatment of THP-1 cells with an antioxidant, N-acetyl cysteine, suppressed the above HNE-induced responses and negated the HNE-mediated increase in TF activity. Blockade of p38 mitogen-activated protein kinase activation inhibited HNE-induced phosphatidylinerse exposure and increased TF activity.

Conclusions—HNE increases TF coagulant activity in monocyctic cells through a novel mechanism involving p38 mitogen-activated protein kinase activation that leads to enhanced phosphatidylinerse exposure at the cell surface. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: 4-hydroxy-2-nonenal ■ atherosclerosis ■ microparticles ■ oxidative stress ■ p38 mitogen-activated protein kinase ■ tissue factor

Reactive oxygen species (ROS) and cellular oxidative stress mediated by lipid peroxidation (LPO) and its products are known major contributors to the initiation and propagation of atherosclerosis. 4-Hydroxy-2-nonenal (HNE) is one of the most abundant reactive aldehydes generated from oxidation of ω6 fatty acids, such as arachidonic acid (AA) and linoleic acid (LA). The concentration of the free form of HNE in the human plasma ranges from 0.1 to ≤1.4 μmol/L and can increase ≥10× during oxidative stress in vivo. It has been shown that HNE can accumulate in the membrane at concentrations ranging from 10 μmol/L to 5 mmol/L in response to oxidative stress and inflammation. HNE is a highly reactive and stable molecule. The amphipathic nature of HNE results in its association with the membranes where it is generated but is also capable of moving within and between cellular compartments and thus can exert its effect on various cellular targets (proteins, lipids, and DNA) far away from its site of origin. HNE can interact covalently with proteins, mainly with the amino (-NH2) groups of lysine and histidine and thiol groups of cysteine, forming Michael adducts, which result in either gain or loss of protein function. Recent studies have demonstrated that HNE contributes to the progression of atherosclerosis through its cytotoxic effects and modulation of various signaling pathways. Several studies using human and animal models have linked HNE with different stages of atherosclerosis. High levels of HNE were found in the atherosclerotic lesions of human subjects and in animal models. HNE can covalently bind to low-density lipoproteins and facilitate their uptake by macrophages. HNE was shown to activate early steps of inflammation and monocyte adhesion to endothelial cells in atherosclerosis.

Tissue factor (TF), a plasma membrane glycoprotein, plays a crucial role in maintaining hemostasis by acting as a cofactor for plasma clotting factor VII (FVII) and activated FVIIa. TF is expressed constitutively on cell surfaces of many extravascular cells, including fibroblasts and pericytes.
in and surrounding blood vessel walls but is absent in cells that come in direct contact with blood, such as monocytes and endothelial cells, under normal physiological conditions.21,22 TF expression can be induced in these cells in response to various pathological stimuli,23,24 and the resultant aberrant expression of TF could lead to intravascular thrombus formation, the precipitating event in acute myocardial infarction, unstable angina, and ischemic stroke.25,26 Although several studies have examined the effects of modified low-density lipoproteins and LPO products on TF expression,27–35 we are not aware of any study that specifically examined the effect of HNE on TF activity.

TF expressed on cells within the atherosclerotic plaque, such as vascular smooth muscle cells, foam cells, monocytes, and endothelial cells overlying atherosclerotic plaques,36–38 is responsible for the thrombogenicity associated with plaque rupture.39,40 Because HNE is a major aldehyde of LPO41 and presence of both HNE and TF has been associated with various stages of atherosclerosis, in the present study, we investigated the effect of HNE on TF expression and its procoagulant function. The data presented in this article show that HNE increases the procoagulant activity of the preexisting TF on cell surfaces through decryption as well as via generation of TF-positive microparticles (MP). Our studies show that HNE-mediated activation of p38 mitogen-activated protein kinase (MAPK) induces exposure of phosphatidylserine (PS) in monocyteic cells without altering lipopolysaccharide (LPS)-induced TF protein levels.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Effect of HNE on TF Activity on Cell Surfaces of Monocytic and Endothelial Cells
Unstimulated THP-1 cells and HCAEC were treated with HNE 20 and 40 µmol/L, respectively, for varying time periods (5 minutes to 24 hours), and TF activity on intact cells was analyzed using FX activation assay. Unperturbed THP-1 cells exhibited low levels of TF activity, and prolonged exposure to HNE (≥24 hours) increased the TF activity by ≥4-fold (data not shown). Treatment of unperturbed THP-1 cells with varying concentrations of HNE revealed that 10 µmol/L HNE was sufficient to significantly increase TF activity. TF activity reached peak with 20 to 40 µmol/L HNE treatment and thereafter declined (Figure 1A). Analysis of TF antigen by immunoblot analysis revealed a faint band (barely visible), corresponding to TF molecular weight (∼50 kDa) in unperturbed THP-1 cells, which did not change in HNE-treated cells (data not shown), indicating that HNE treatment did not induce TF protein expression. Unperturbed HCAEC expressed no measurable TF activity, and HNE treatment (40 µmol/L for 4 hours) did not induce TF activity. Similarly, no TF antigen was found in unperturbed HCAEC, either untreated or treated with HNE (data not shown).

Because the above data suggest that HNE does not induce TF protein but enhances the activity of TF that is already present on the cell surface, we next examined the effect of HNE on monocytic and endothelial cells that are perturbed to induce TF protein. Before using perturbed THP-1 cells as a model system to investigate the effect of HNE, we first examined whether HNE influences TF activity of monocyte-derived macrophages. LPS-perturbed monocyte-derived macrophages were exposed to HNE (40 or 80 µmol/L) for varying times (15 minutes to 6 hours), and TF coagulant activity on intact cells was determined using FX activation assay. Both concentrations of HNE enhanced TF activity by 4- to 5-fold at 3 or 6 hours of HNE treatment (data not shown). Next, LPS-perturbed monocyte-derived macrophages were treated with varying concentrations of HNE for 4 hours. HNE treatment significantly increased TF activity in LPS-perturbed macrophages in a dose-dependent manner, reaching optimum at 40 µmol/L of HNE (3.5-fold increase over LPS alone–treated macrophages; Figure I in the online-only Data Supplement). Next, we examined whether this effect of HNE could be mimicked in THP-1 cells. As observed with monocyte-derived macrophages, HNE treatment markedly increased cell surface TF activity of LPS-perturbed THP-1 cells in a dose-dependent (Figure 1B) and time-dependent manner (Figure 1C). A concentration of 20 to 40 µmol/L HNE exerted the maximal effect, a 4- to 5-fold increase in TF activity over LPS alone–treated cells.

HNE treatment also increased TF activity in cytokine-perturbed HCAEC (Figure 1D and 1E) and human umbilical vein endothelial cells (data not shown). A 2-fold increase in TF activity was observed at 20 µmol/L HNE, and increasing the HNE concentration to 40 to 80 µmol/L further enhanced the TF activity (3-fold) in HCAEC (Figure 1D). Treatment of HNE-treated THP-1 cells or HCAEC with inhibitory TF monoclonal antibody (5G9) completely inhibited FX activation, indicating that HNE-mediated increase in cell surface coagulant activity was TF specific (data not shown). Additional studies revealed that the concentrations of HNE and treatment durations used in above experiments had no significant effect on cell viability as measured using trypan blue dye exclusion method or MTT assay (Figure II in the online-only Data Supplement).

Effect of Other LPO Products on TF Activity
We next performed a comparative evaluation of various fatty acids and aldehydes generated in the process of LPO for their potential to induce TF activity in LPS-stimulated THP-1 cells and tumor necrosis factor-α+interleukin 1-β–perturbed HCAEC. THP-1 cells and HCAEC were treated with 20 and 40 µmol/L, respectively, of HNE, 4-hydroxy hexenal, 2,4-decadional (DDE), acrolein, malondialdehyde, AA, LA, and methylglyoxal, and cell surface TF activity was measured. As shown in Figure 1F, apart from HNE, only DDE treatment significantly increased TF activity in THP-1 cells. In case of HCAEC, 4-hydroxy hexenal, acrolein, and DDE induced TF activity to a similar or slightly lower extent to that of HNE (Figure 1G). Here, it may be pertinent to note that HNE being the most abundant and reactive molecule formed during LPO compared with others,9,41 it may be a more physiologically relevant molecule to affect TF procoagulant activity. Therefore, we used only HNE in further studies.
HNE Increases TF Procoagulant Activity Without Increasing TF Protein Levels

To determine whether HNE-mediated increase in TF activity in LPS-stimulated THP-1 cells is a result of further increase in TF antigen, we quantitatively measured both surface and total TF antigen levels in THP-1 cells treated with a control vehicle or HNE. As shown in Figure 2A, cell surface binding analysis performed using radiolabeled TF monoclonal antibody (TF 9C3) showed no differences in cell surface TF antigen levels between control vehicle- and HNE-treated THP-1 cells. Similarly, no differences were found in total TF antigen levels between control and HNE-treated THP-1 cells as determined by ELISA (Figure 2B). These data show, as observed with unperturbed cells, that HNE treatment does not increase TF antigen levels in LPS-stimulated THP-1 cells. This indicates that HNE-mediated increase in TF activity did not stem from increased TF protein levels on the cell surface.

HNE Does Not Modify TF Protein but Increases Exposure of PS on Cell Surfaces

HNE is known to form adducts with several proteins by binding to lysine, histidine, and cysteine amino acid residues and thus modulating their function and activity. Therefore, we evaluated the effect of HNE on TF activity in a buffer system containing relipidated purified TF. Because HNE is known to bind to PE, we analyzed the effect of HNE on TF relipidated in either PC/PS or PC/PS/PE vesicles. HNE treatment (20 µmol/L for 4 hours) had no effect on the activity of TF relipidated in either PC/PS or PC/PS/PE vesicles (data not shown). Furthermore, Western blot analysis with HNE

Figure 1. 4-hydroxy-2-nonenal (HNE)–mediated increase in tissue factor (TF) procoagulant activity in THP-1 cells and HCAEC and its comparison with other reactive aldehydes. THP-1 cells or HCAEC were treated with HNE and other compounds as described below, and at the end of treatment, cell surface TF activity was determined using factor X (FX) activation assay. A, Unperturbed THP-1 cells were treated with varying concentrations of HNE for 4 hours. B, THP-1 cells were stimulated with 1 μg/mL lipopolysaccharide (LPS) for 4 hours in RPMI medium containing 2% serum and then treated with varying concentrations of HNE for 4 hours. C, LPS-stimulated THP-1 cells were treated with 20 µmol/L HNE for varying time periods. D and E, HCAEC monolayers cultured in 48-well plate were perturbed with 20 ng/mL of each tumor necrosis factor (TNF)-α and interleukin (IL) 1-β for 4 hours in 2% serum containing EBM-2 medium and then treated with varying concentrations of HNE for 4 hours (D) or with 40 µmol/L HNE for varying time periods (E). F and G, LPS-stimulated THP-1 cells (F) or cytokine-activated HCAEC (G) were treated with HNE and other reactive aldehydes for 4 hours (20 µmol/L concentration for THP-1 cells and 40 µmol/L concentration for HCAEC). In A, * denotes that the value is statistically significantly higher compared with cells not treated with HNE (P<0.05). In other panels, # denotes statistically significantly difference from unperturbed cells (P<0.01); * denotes statistically significant difference compared with unperturbed cells that were subjected to HNE or other treatments (P<0.05). AA indicates arachidonic acid; DDE, 2,4-decadional; LA, linoleic acid; and UN, unperturbed cells.
antibodies failed to detect TF–HNE complex. Similarly, HNE antibodies failed to detect TF–HNE complex in TF immunoprecipitate of cell lysates of LPS-perturbed THP-1 cells treated with HNE (data not shown). Immunoblot analysis of whole cell lysates of LPS-perturbed THP-1 cells treated with HNE showed several HNE–protein adducts, mostly at higher molecular weight (>60 kDa), which ran as a smear on the gel. Overall, these data indicate that it is unlikely that potential modification of amino acids on TF by HNE is responsible for the increased TF activity observed in cell systems.

It has been reported that on cell surfaces only small amounts of TF are coagulant active (capable of activating FX), whereas majority of the TF is inactive (cryptic). Several mechanisms have been proposed regarding transformation of cryptic TF into procoagulant active TF, but the exposure of PS on the cell surface in response to various stimuli has been widely accepted. To test whether HNE-mediated increase in TF activity on the cell surface is a result of increased PS at the cell surface in HNE-treated cells, we investigated the effect of annexin V, a protein known to bind PS, on HNE-mediated increased TF activity. As shown in Figure 2C, blocking anionic phospholipids on the cell surface with annexin V abrogated HNE-mediated increased TF activity. Consistent with the possibility that HNE treatment enhances PS exposure to the outer leaflet, treatment of THP-1 cells, either unperturbed or LPS perturbed, with HNE significantly increased the prothrombinase activity on the cell surface (Figure 2D). As observed with TF activity, annexin V completely inhibited HNE-mediated increase in prothrombinase activity (data not shown). In additional studies, we analyzed the effect of HNE on transmembrane transport of PS in THP-1 cells using NBD-PS. As shown in Figure 2E, ≈70% to 90% of NBD-PS associated with the cell surface was internalized in unperturbed or LPS-stimulated THP-1 cells over 10 minutes, and HNE treatment significantly decreased the uptake of NBD-PS. These data indicate that HNE inhibits the PS influx. Overall, these data suggest that HNE treatment increases the exposure of anionic phospholipids at the outer leaflet and that increased anionic phospholipids on the cell surface is primarily responsible for the increased TF activity on HNE treatment.

**HNE-Induced ROS Generation and Its Potential Role in Increasing the Activity of TF**

HNE was shown to stimulate ROS generation in different cell types. Therefore, we investigated whether HNE leads to generation of ROS in THP-1 cells and HCAEC and whether
generation of ROS is responsible for HNE-mediated increase in TF activity. To determine this, both unperturbed and perturbed THP-1 cells and HCAEC were treated with HNE for 1 hour and ROS production was measured by H$_2$DCFDA oxidation. As shown in Figure 3A and 3B, HNE treatment significantly enhanced ROS production in both unperturbed and perturbed THP-1 cells and HCAEC. Next, to examine the possible source of HNE-mediated ROS generation, perturbed THP-1 cells and HCAEC were treated with inhibitors of mitochondrial electron transport chain (rotenone), xanthine oxidase (allopurinol), and NAPDH-oxidase (apocynin) before HNE treatment. Rotenone inhibited HNE-induced ROS generation significantly, but not completely, in THP-1 cells (Figure 3A) but had no effect in HCAEC. Other inhibitors had no noticeable effect on the HNE-induced ROS generation, either in THP-1 cells or HCAEC (data not shown). As shown in Figure 3C, of all the inhibitors tested, only rotenone showed significant (≈50% inhibition) HNE-mediated increase in TF activity in THP-1 cells. These results suggest that HNE-induced mitochondrial ROS production contributes partially but significantly to HNE-mediated activation of TF activity in THP-1 cells. In contrast to THP-1 cells, none of the inhibitors was effective in attenuating HNE-mediated increase in TF activity in HCAEC (Figure 3D).

Involvement of p38 MAPK Activation in HNE-Mediated Increase of TF Activity Through Regulation of PS Exposure

HNE is known to activate MAPK signaling pathways. Recent studies indicate that p38 MAPK activation may play a role in PS exposure in erythrocytes and platelets. Therefore, to elucidate the putative signal transduction mechanism responsible for activation of TF by HNE, we examined the effect of specific MAPK inhibitors on HNE-mediated increase in TF activity (Figure 4A and 4B). As shown in Figure 4A, preincubation of cells with p38 MAPK inhibitor (SB203580), before addition of HNE, completely attenuated HNE-mediated activation of TF, whereas inhibitors of JNK (SP600125) and ERK (PD98059) were without any effect. We further confirmed the involvement of p38 MAPK signaling in HNE-mediated effect by immunoblot analysis of p38 MAPK activation. As shown in Figure 4C, HNE treatment increased phosphorylation of p38 MAPK, which was attenuated by the p38 MAPK inhibitor (SB203580), whereas JNK and ERK inhibitors had no effect on p38 MAPK activation. Here, it may be pertinent to note that although LPS treatment, in itself, activates p38 MAPK to some extent, phospho p38 MAPK levels declined to basal levels by the time cells were subjected to HNE treatment (Figure III in the online-only Data Supplement). HNE activation of p38
MAPK was not dependent on LPS because HNE was found to activate p38 MAPK in unperturbed THP-1 cells (Figure III in the online-only Data Supplement). HNE-induced phospho p38 MAPK levels were sustained for $\geq 4$ hours in THP-1 cells (Figure III in the online-only Data Supplement).

To demonstrate that HNE-induced p38 MAPK activation is responsible for exposure of PS in THP-1 cells, we performed fluorescence staining of THP-1 cells treated with HNE±SB203580 with AF488-annexin V to evaluate PS exposure on the cell surface. As shown in Figure 4D, HNE treatment increased the number of cells brightly stained with AF488-annexin V and pretreatment of cells with p38 MAPK inhibitor markedly reduced AF488-annexin V staining. These data provide strong evidence that HNE-led activation of p38 MAPK signaling is responsible for PS exposure on the cell surface. Interestingly, in contrast to THP-1 cells, p38 MAPK inhibitor had only a negligible effect on HNE-dependent TF activation in HCAEC (Figure 4B), indicating that an alternate signaling pathway may exist for HNE-mediated TF activation in endothelial cells.

Thiol Protective Agents Attenuate HNE-Mediated ROS Generation, p38 MAPK Activation, and Increase in TF Activity

The glutathione (GSH)/GSH S-transferase system is the most dominant system involved in the rapid detoxification and disposal of HNE from cells.46 N-acetyl cysteine (NAC) and N-2 mercaptopropionyl glycine are known antioxidants and thiol protectants that exert protective effects by replenishing cellular GSH. This prompted us to examine whether NAC can attenuate HNE-mediated cellular ROS generation, p38 MAPK activation, and increase in TF activity. As shown in Figure 5A and SB, pretreatment of perturbed THP-1 cells or HCAEC with NAC completely abrogated HNE-induced ROS generation. NAC and mercaptopropionyl glycine also inhibited HNE-induced p38 MAPK activation in THP-1 cells (Figure 5C) and increase in TF activity on the cell surface in both perturbed THP-1 cells (Figure 5D) and HCAEC (Figure 5E). In additional studies, we examined the effect of NAC on HNE-induced PS exposure on the cell surface in THP-1 cells. Analysis of PS exposure on the cell surface by AF488-annexin V binding to intact THP-1 cells showed that NAC completely attenuated HNE-induced PS exposure on the cell surface without affecting TF antigen levels (Figure 5F).

Rotenone, an inhibitor of mitochondrial electron transport chain, which partly but significantly inhibited HNE-induced ROS generation and TF activity (Figure 3), had no effect on HNE-induced p38 MAPK activation (data not shown). However, rotenone partly suppressed HNE-induced PS exposure on the cell surface (Figure 5G; % annexin V–positive cells in LPS-stimulated THP-1 cells treated with HNE, 15.1±1.2; in cells pretreated with rotenone before adding HNE, 8.9±1.2; $P=0.0026$). These data indicate that HNE-induced p38 MAPK...
activation may be independent of HNE-induced ROS generation via mitochondrial respiratory complex I, but the later process could also contribute to externalization of PS to some extent.

HNE Releases TF-Bearing Microparticles From Endothelial Cells and Fibroblasts

TF decryption results in increased TF activity on the cell surface, as well as generation of highly procoagulant TF-positive microparticles. Therefore, we investigated whether apart from activating cell surface TF, HNE can induce production of TF-bearing microparticles. HNE treatment of perturbed HCAEC (Figure 6A) and WI-38 fibroblasts (Figure 6B) significantly increased TF activity associated with microparticles in a time-dependent manner. Surprisingly, although activation of monocytes/macrophages by various agents was shown to generate procoagulant microparticles, HNE treatment of LPS-perturbed THP-1 cells did not lead to further enhancement of microparticle-associated TF activity compared with LPS alone–treated cells (data not shown).

Discussion

Although various LPO products were shown to induce TF protein expression and activity in monocytes and endothelial cells, the effect of HNE, one of the most abundant aldehydes formed during LPO of ω-6 polyunsaturated fatty acids, on TF coagulant function has not been investigated. Here, we show for the first time that (1) HNE increases the procoagulant activity of TF in perturbed monocytic and endothelial cells without increasing TF antigen levels; (2) HNE-induced mitochondrial ROS generation is partially responsible for increased TF activity in monocytic cells; (3) HNE-induced activation of p38 MAPK pathway followed by PS exposure is responsible primarily for increased TF activity in monocytic cells; (4) treatment of monocytic and endothelial cells with thiol protectants attenuates HNE-mediated PS exposure and increased TF activity on the cell surface; and (5) HNE increases the generation of TF-bearing microparticles from perturbed endothelial cells and fibroblasts.
Although TF is essential for maintaining hemostasis, the aberrant expression of TF induced by various disease conditions results in intravascular thrombotic complications.\(^{51,52}\) Oxidative stress and LPO contribute to various cardiovascular diseases, including atherosclerosis.\(^{53–56}\) Several studies have examined the effects of modified low-density lipoprotein on TF expression, which failed to provide conclusive data because they showed increase, no change, or even inhibition of TF expression.\(^{27,29–35}\) Aldehydes are the end products of polyunsaturated fatty acid oxidation and have been shown to play a key role in the progression of atherosclerotic process by modifying many important biological functions.\(^{57}\) The best known effect of HNE and other reactive aldehydes is their reaction with Lys residues of apolipoprotein B, which leads to the recognition of oxidized low-density lipoprotein by the scavenger receptors.\(^{57}\) This seems to be a predominant mechanism by which LPO end products contribute to the progression of atherosclerosis.\(^{1,58}\) However, HNE can also modulate various cellular functions that contribute to atherosclerotic process by directly interacting with biomolecules (proteins, lipids, and DNA), activation of stress signaling pathways, and inducing intracellular oxidative stress.\(^{27–56}\) Earlier studies showed that LPO and its end products can modulate TF expression and procoagulant activity in various cell systems, mostly inducing TF mRNA and protein synthesis.\(^{7,8,49,50,60}\) Cabré et al.\(^{28}\) showed that hexenal and DDE, 2 other aldehydes produced in LPO, increased TF expression by transcriptional activation of TF gene in vascular smooth muscle cells. In the present study, we find that HNE increases TF procoagulant activity in LPS-stimulated THP-1 cells and cytokine-stimulated HCAEC without increasing TF antigen. Of all biologically relevant aldehydes we tested, HNE seems to be the most potent in increasing TF activity and DDE was also found to effectively increase TF activity in both THP-1 cells and HCAEC. However, in addition to HNE, DDE was also found to effectively increase TF activity of both perturbed monocytes and endothelial cells. It may be pertinent to note here that although DDE may be more biologically active it is least abundant in plasma.\(^{28,61}\) In earlier studies, AA and LA, the precursors of HNE, were shown to either enhance TF protein and activity\(^{49}\) or inhibit LPS-induced TF expression in macrophages.\(^{60}\) In the present study, we found that both AA and LA had no significant effect on TF activity in THP-1 cells as they constitutively express low levels of TF antigen. In the above studies, the increased TF activity and PS exposure in cells treated with cytotoxic chemotherapeutics seemed to be associated with apoptosis. Although HNE also induces apoptosis, under our experimental conditions there was no significant decrease in the number of viable cells after treatment with HNE, indicating that HNE-induced PS externalization and increased TF activity observed in the present study may be specific and distinct from PS externalization that occurs during apoptosis.

HNE has been shown to generate ROS,\(^{3,6,7}\) which is known to modulate many cellular functions by multiple signaling pathways.\(^{68}\) ROS can be generated through several sources viz. NADPH oxidase, xanthine oxidase, mitochondrial respiratory complex I and III, and nitric oxide synthase.\(^{69}\) Earlier studies showed that ROS generated by xanthine:xanthine oxidase or copper metal complex increased TF activity by increasing the synthesis of TF mRNA,\(^{70,71}\) whereas the oxidant \(\text{H}_2\text{O}_2\) increased TF activity by activating the latent TF without increasing TF mRNA.\(^{72}\) The effect of HNE on TF seems to be
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similar to that of $\text{H}_2\text{O}_2$, that is, activating the latent TF on the cell surface. It is pertinent to note here that HNE was shown to strongly induce peroxide production and the peroxide is thought to play a crucial role in HNE-induced stress signaling pathways. The observation that rotenone, an inhibitor of mitochondrial respiratory complex I and not the inhibitors of NADPH oxidase or xanthine oxidase, inhibited increase in HNE-induced TF activity in THP-1 cells suggests that HNE-induced ROS generated via mitochondrial respiratory complex I may play a role in HNE-mediated TF activation in THP-1 cells. Because rotenone completely blocks neither the HNE-induced ROS generation nor TF activity increase, other cellular pathways may also contribute toward HNE-mediated ROS generation and TF activation in THP-1 cells. Rotenone was shown to completely inhibit HNE-induced ROS generation in bovine lung microvascular endothelial cells. However, in the present study, rotenone as well as other inhibitors tested failed to attenuate HNE-mediated ROS production and TF activation in HCAEC. This discrepancy in results could be a result of potential differences in the cell models used.

Recently, Canault et al showed that inhibition of p38 MAPK signaling prevented PS exposure and microparticle formation in the stored platelets. Because HNE is known to activate p38 MAPK, JNK, and ERK pathways, we investigated the involvement of these pathways in HNE-mediated increased TF activity. Our data reveal that HNE-mediated p38 MAPK pathway activation is responsible for PS exposure because p38 MAPK inhibitor and not JNK and ERK pathway inhibitors markedly attenuated PS exposure in THP-1 cells. Consistent with the hypothesis that HNE-induced PS exposure via p38 MAPK pathway is responsible for increased TF activity in HNE-treated cells, treatment of monocytic cells with the p38 MAPK inhibitor markedly reduced the increase in HNE-induced TF activity in THP-1 cells. At present, it is unclear whether HNE-induced ROS generation plays a role in p38 MAPK activation because rotenone, which inhibited the HNE-induced ROS generation significantly, but not completely, failed to suppress HNE-induced p38 MAPK activation. In contrast to that observed in THP-1 cells, p38 MAPK inhibitor had no effect on HNE-mediated increased TF activity in HCAEC, indicating that the mechanism by which HNE increases TF activity in endothelial cells may differ from that of monococytes.

In addition to the above described mechanisms, HNE can also modulate various cellular functions by depleting the cellular GSH. GSH is an important intracellular antioxidant and its depletion is associated with cardiovascular diseases. HNE generated in cells can be detoxified by GSH S-transferases or by thiol protectants, such as NAC and mercaptopropionyl glycine. Complete abolishment of HNE-mediated increase in TF activity by NAC and mercaptopropionyl glycine suggests that cellular GSH levels regulate TF activity indirectly. NAC was also shown to inhibit functional and antigenic expression of TF in human monocytes. Because cells were treated with NAC after 3 hours of stimulation with LPS and NAC treatment had no effect on LPS-induced TF expression per se, our data suggest that NAC inhibited HNE-mediated effects through its thiol protectant ability to detoxify HNE by forming GS-HNE.

Microparticles shed by activated and apoptotic cells play a major role in the development and progression of atherosclerosis and other cardiovascular diseases. MP derived from vascular cells, blood peripheral cells, as well as apoptotic cells sequestered within the atherosclerotic plaque are known to be reservoirs of TF activity, which can promote coagulation after plaque rupture. TF MPs are detected in patients with atherosclerosis. Similarly, increased concentrations of HNE were found in atherosclerotic lesions. These findings coupled with our present observation that HNE increases the generation of TF-bearing microparticles suggest that HNE can play a crucial role in the development of thrombotic disorders associated with atherosclerosis by release of TF-positive MP from endothelial cells that are activated by pathological stimuli or smooth muscle cell and fibroblasts that constitutively express TF. HNE is also known to impair endothelial barrier function by downregulating cell adhesion proteins. Under such compromised conditions, MPs released from underlying fibroblasts can come into the circulation and activate systemic thrombosis.

In summary, the results presented herein show that HNE, one of the most abundant reactive aldehydes generated from oxidation of $\text{o}6$ fatty acids, is capable of increasing the activity of TF in both monocytes and endothelial cells. HNE induces PS externalization, which is responsible for increased TF activity on the cell surface, in monocytic cells through a novel mechanism involving the activation of p38 MAPK pathway. Further studies are needed to elucidate intracellular signaling pathways that lead HNE-induced p38 MAPK activation to PS externalization and to determine the relevance of HNE-induced TF activation in thrombotic complications associated with atherosclerosis and other diseases where HNE can be produced and to elucidate mechanistic details.

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Disclosures

None.

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**Significance**

This study identifies a novel mechanism by which a lipid peroxidation product, 4-hydroxy-2-nonenal, enhances the activity of a procoagulant factor, tissue factor, on cell surfaces of mononuclear and endothelial cells. These findings may have relevance in understanding how reactive products generated in atherosclerosis could cause thrombotic disorders.
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Figure 1. HNE increases TF procoagulant activity in human monocyte-derived macrophages (MDMs). MDMs cultured in 48-well plate were stimulated with LPS (1 µg/ml) for 4 h in RPMI medium containing 2% human serum. After 4 h, varying concentrations of HNE were added to cells, and the cells were incubated for an additional 4 h. At the end of the treatment, cells were washed with buffer A and cell surface TF activity was determined in a FX activation assay using 10 nM FVIIa and 175 nM FX. Data are mean ± SEM (n = 4-6). UN, unperturbed cells; # denotes statistical significant difference compared to unperturbed cells (p <0.01); * denotes statistical significant difference compared to LPS-stimulated cells that were not subjected to HNE treatment (p <0.02).
Figure II. Cytotoxicity measurement in HNE-treated THP-1 cells and HCAEC. (A) LPS (1 µg/ml)-perturbed THP-1 cells (2 x 10⁵/ml) or (B) TNF-α + IL1-β (20 ng/ml)-perturbed HCAEC in 96-well plate were treated with varying concentrations of HNE for 4 h at 37°C. In the last 2 h of HNE treatment, MTT reagent (250 µg/ml) was added to the cells. At the end of the treatment, overlying medium was removed, cells were dissolved in 100 µl of DMSO with gentle shaking for 1 h at room temperature, and then absorbance at 562 nm was measured using a microplate reader. ns, not statistically significant differences; # denotes statistical significant difference compared to cells not treated with HNE (p < 0.05).
Figure III. Kinetics of HNE-induced p38 MAPK phosphorylation. (A) Unperturbed THP-1 cells were treated with HNE (20 µM) for varying time periods as indicated in the figure. (B) THP-1 cells were treated with LPS (1 µg/ml) for varying time periods. (C) THP-1 cells were first stimulated with 1 µg/ml LPS for 4 h, and then treated with HNE (20 µM) for varying time periods. At the end of the treatment, cells were lysed and subjected to a non-reducing SDS-PAGE and immunoblotted with phospho p38 and total p38 MAPK antibodies.
4-Hydroxy-2-nonenal Enhances Tissue Factor Activity in Human Monocytic Cells via p38 MAPK Activation-dependent Phosphatidylserine Exposure

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Materials and Methods

Reagents
HNE, AA, LA, and 4-hydroxy hexenal were purchased from Cayman Chemical (Ann Arbor, MI). 2,4-decadional was from Acros Organics (Morris Plains, NJ), Acrolein was from Chem Service (West Chester, PA), and methylglyoxal was from MP Biomedicals (Solon, OH). Recombinant human factor VIIa (FVIIa) was from Novo Nordisk (Gentofte, Denmark). Purified human factor X, Xa, and prothrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Purified human alpha-thrombin and factor Va were purchased from Hematologic Technologies, Inc (Essex Junction, VT). Mono-specific polyclonal antibodies against human TF were prepared as described earlier.1 TF hybridomas (9C3 and 5G9) were kindly provided by James H. Morrissey, University of Illinois College of Medicine, Urbana, IL. Antibodies against phospho p38 MAPK and total p38 MAPK were obtained from Cell Signaling Technology Inc. (Danvers, MA). Annexin V was kindly provided by Jonathan F. Tait (University of Washington, Seattle, WA). Cell culture medium DMEM, RPMI 1640, and fetal bovine serum were from GIBCO (Invitrogen, Carlsbad, CA). Secondary fluorescence conjugated antibodies, AF488 conjugated annexin V, and H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) were from Life Technologies, Grand Island, NY. NBD-PS (1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphoserine) and other phospho lipids were obtained from Avanti Polar Lipids (Alabaster, AL). TNF-α and IL1-β were obtained from R&D Systems (Minneapolis, MN). LPS and other reagents were purchased from Sigma–Aldrich (St. Louis, MO).

Cell Culture
Primary human coronary artery endothelial cells (HCAEC), umbilical vein endothelial cells (HUVEC), EBM-2 basal medium, and endothelial cell growth supplements were purchased from Lonza (Walkersville, MD). Endothelial cells were cultured in EBM-2 basal medium supplemented with the growth supplements and 5% fetal bovine serum. Endothelial cell passages between 3 and 7 were used in the present studies. THP-1 cells and WI-38 fibroblasts were purchased from ATCC (Rockville, MD) and grown in RPMI 1640 and DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin, respectively.

Isolation and culture of monocyte-derived macrophages (MDMs)
Blood (60 ml) was collected from healthy volunteers by venipuncture into BD Vacutainer containing sodium heparin (final heparin concentration 15 U/ml). Blood was diluted 1:1 with Hank’s balanced salt solution (with no CaCl2, MgCl2 or phenol red). Peripheral blood mononuclear cells (PBMCs) were isolated by differential centrifugation over Ficoll-Paque (GE Healthcare, density 1.0776 ± 0.001 g/ml). The isolated PBMCs were plated in culture plates in RPMI containing 10% heat-inactivated human serum AB type (Atlanta Biologicals, Lawrenceville, GA) and sodium pyruvate and allowed to adhere for 2 h. Non-adherent cells and loosely adhered cells were removed by aspiration and washing the adhered cells gently 2–3 times with warm RPMI serum free medium. Subsequently, adherent monocytes were allowed to
mature into macrophages by culturing in RPMI complete medium for 4–5 days at 37°C in a humidified 5% CO2 atmosphere.

**MTT assay**

Cell viability of THP-1 and HCAEC in the presence of HNE was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay essentially as described by Mosmann, and detailed in our earlier publication.

**Relipidation of TF**

Eukaryotic cell expressed recombinant TF containing transmembrane spanning domain (TF1-248) (130 nM) was incubated with 1.3 mM PC/PS (80% 1,2-dioleoyl-sn-glycero-3-phosphocholine and 20% 1,2-dioleoyl-sn-glycero-3-phospho-L-serine) or PC/PS/PE (60% PC, 20% PS, and 20% PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanol amine) in HEPES buffer (10 mM HEPES, 0.15 M NaCl, pH 7.5) containing 40 mM octyl β-D-glucopyranoside for 60 min at 37°C. The relipidated TF was subjected to extensive dialysis in HEPES buffer at 4°C to remove the detergent.

**Measurement of tissue factor activity**

Confluent monolayers of HCAEC (or HUVEC) cultured in 48-well plate or THP-1 cells (2 X 10^5/ml) in suspension were incubated with FVIIa (10 nM) in buffer B (buffer A [10 mM HEPES, 0.15 M NaCl, 4 mM KCl, 11 mM glucose, pH 7.5] containing 5 mM CaCl₂, 1 mM MgCl₂ and 1 mg/ml BSA) for 5 min at 37°C followed by the addition of FX (175 nM) to initiate TF-FVIIa-mediated activation of factor X. An aliquot was removed after 10 min into the stop buffer (Tris buffered saline [TBS; 20 mM Tris.HCl, 0.15 M NaCl, pH 7.5] containing 1 mg/ml BSA and 10 mM EDTA), and the amount of FXa generated was measured in a chromogenic assay using Chromozym S-2765 as described earlier. A similar FX activation assay was used to measure the activity of relipidated TF subjected to HNE treatment.

**Determination of prothrombin activation on cell surfaces**

THP-1 cells (2 X 10^5/ml) in suspension were incubated with buffer B containing FVa (10 nM) and FXa (1 nM) for 5 min at 37°C. Subsequently, prothrombin (5 μM) was added to the cells and at the end of 1 min activation period, reaction was stopped with TBS buffer containing 10 mM EDTA. Thrombin generated was measured in a chromogenic assay using Chromozym TH as described earlier.

**Radiolabeling of TF mAb and determination of TF levels at the cell surface**

TF mAb was labeled with 125I using IODOGEN (Pierce Biotechnology, Rockford, IL, USA)-coated polypropylene tubes and Na 125I (Perkin Elmer Life Sciences, Wellesley, MA, USA) according to the manufacturer's protocol. Briefly, 100 μg of TF mAb was incubated with Na 125I (1 mCi) in a 10 μg of IODOGEN-coated tube on ice for 20–30 min, and reaction was stopped by the addition of 1% potassium iodide. The sample was then dialyzed for 18–20 h in HEPES buffer at 4°C to remove free iodide. 2 X 10^6 THP-1 cells were stimulated with LPS (1 μg/ml) for 4 h in RPMI medium containing 2% serum. At the end of 4 h, 20 μM of HNE was added to the cells and incubated for another 4 h. Cells were then washed twice with buffer A and chilled on ice for 10 min and incubated with radiolabeled TF mAb (10 nM) for 2 h at 4°C. Thereafter, supernatant was removed and cells were washed four times with ice-cold buffer B to remove unbound radiolabeled TF mAb. The surface-bound radiolabeled TF mAb was eluted by adding low pH glycine (0.1 M, pH 2.3) for 5 min. Eluted radioactivity was then counted in a γ-counter.
**Immunoblotting**
After specified treatments, cells were washed with PBS and lysed in SDS-PAGE (non-reducing) buffer. Immunoblot analysis was performed by loading 10-20 μl of cell extracts on 12% SDS-PAGE, transferring onto PVDF membrane and probing with specific antibodies. The same blots were also probed with anti-GAPDH for GAPDH loading control. To determine whether HNE forms adduct with TF in THP-1 cells, cell lysates of LPS-activated THP-1 cells (1 x 10^6 cells/600 μl of 2% Triton X-100 in TBS) treated with control vehicle or HNE for 4 h were immunoprecipitated with 0.5 mg of Dynabeads coupled with TF mAb (9C3, 10 μg TF mAb/mg beads). TF was eluted from the immunoprecipitate with 0.1 M glycine, pH 2.3 and the eluates were subjected to western blot analysis with TF or HNE antibodies. Immunoprecipitation was omitted when analyzing for potential modification of relipidated TF by HNE.

**TF ELISA**
Total TF antigen was measured in an ELISA as described earlier with slight modifications. TF monoclonal antibody 9C3 was used as the capture antibody and biotinylated rabbit anti-TF IgG was used as the detecting antibody.

**Measurement of ROS generation**
ROS generation was measured by analyzing the conversion of non-fluorescent H_{2}DCFDA loaded onto cells to fluorescent dichlorofluorscein (DCF) following its oxidation. Briefly, cells were loaded with H_{2}DCFDA (for THP-1 cells, 5 μM for 10 min; HCAEC, 10 μM for 30 min) in serum-free medium. After removing the supernatant medium containing H_{2}DCFDA, cells were washed once with serum-free-medium, and treated with HNE and other compounds as described in the figure legends. The fluorescence of cells was viewed using laser wavelength settings of 488 ± 10 nm for excitation and 525 ± 10 nm for emission, and digital images of the fluorescence were captured using LSM 510 Meta confocal system (Zeiss) equipped with Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany). DIC images of the same fields were also obtained. The percent of cells emitting fluorescence was determined by counting the total number of cells (from DIC images) and the number of fluorescent cells from multiple fields. To quantify the fluorescence intensity, a region of interest (ROI) was made encircling individual cells and measuring the fluorescence intensity within the ROI using ZEN 2009 software (Carl Zeiss). At least 50 or more cells from two or more fields were used for quantification.

**Analysis of PS dynamics**
Transbilayer movement of PS was measured using NBD-PS as described previously. To measure the effect of HNE on transfer of PS from the outer leaflet to inner leaflet, first THP-1 cells (1 x10^6 cells/ml), unstimulated or LPS-stimulated, were exposed to HNE (20 μM) for 30 min at room temperature. At the end of incubation, cells were washed twice and incubated with ice-cold incubation buffer (buffer A containing 500 μM PMSF, pH 7.5) for 5 min. Then, NBD-PS (2 μM) was added to the cells, and allowed to incubate for 10 min at 4°C. At the end of 10 min, the cells were washed twice with ice-cold buffer, then the cells were suspended in the same buffer that was prewarmed to 37°C (1 x 10^6 cells/ml), and the cell suspension was incubated at 37°C. At varying time intervals, cell aliquots (200 μl) were removed, and fluorescence intensity of cells was measured in the presence and absence of cell impermeant reducing agent, dithionite (25 μM) that quenches the NBD-PS exposed on the cell surface. Varian Cary Eclipse fluorescence spectrophotometer equipped with Peltier thermocontroller (Varian Inc.) was used for fluorescence measurements. The percent NBD-PS internalized was determined as the percentile of dithionite-resistant NBD-PS fluorescence from the total NBD-PS fluorescence as described earlier.
Immunofluorescence confocal microscopy
Unstimulated and LPS-stimulated THP-1 cells at the end of treatments were washed with phosphate buffered saline. The cells were then fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100 and processed for immunostaining for TF as described earlier.\textsuperscript{5} Nuclei were stained with DAPI. After completion of immunostaining, slides for microscopy were made by subjecting the cells for cytopinning. The fluorescence of immunostained cells were viewed, and digital images of fluorescence were captured using LSM 510 Meta confocal system (Zeiss) equipped with Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany) at 40x or 63x magnification (oil) plan-apochromate lens. The laser setting wavelengths were 369 ± 10 nm excitation and 450 ± 30 nm emission for DAPI and 488 ± 10 nm excitation, 525 ± 10 nm emission for AF488 conjugated annexin V and 543 ± 10 nm excitation and 575 ± 10 nm emission for Rhodamine Red. The images were processed using LSM software Zen 2009 (Zeiss) and imported to Adobe Photoshop (vs. 7.0) for compilation of figures.

Data collection and statistical analysis
Data shown in the figures represent mean ± SEM (n = 3 to 5) or a representative image. Student unpaired t-test was employed for determining statistical significance.

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