Mitogenic Responses of Vascular Smooth Muscle Cells to Lipid Peroxidation-derived Aldehyde 4-Hydroxy-trans-2-nonalen (HNE)

ROLE OF ALDOSE REDUCTASE-CATALYZED REDUCTION OF THE HNE-GLUTATHIONE CONJUGATES IN REGULATING CELL GROWTH*

Received for publication, January 10, 2006, and in revised form, April 26, 2006. Published, JBC Papers in Press, April 28, 2006, DOI 10.1074/jbc.M600270200

Kota V. Ramana‡, Aruni Bhatnagar‡, Sanjay Srivastava‡, Umesh C. Yadav‡, Sanjay Awasthi‡, Yogesh C. Awasthi†, and Satish K. Srivastava‡,‡

From the ‡Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas 77555, the †Division of Cardiology, Department of Medicine, University of Louisville, Louisville, Kentucky 40202, and the ¤Department of Chemistry & Biochemistry, University of Texas, Arlington, Texas 76019-0065

Products of lipid peroxidation such as 4-hydroxy-trans-2-nonalen (HNE) trigger multiple signaling cascades that variably affect cell growth, differentiation, and apoptosis. Because glutathiolation is a significant metabolic fate of these aldehydes, we tested the possibility that the bioactivity of HNE depends upon its conjugation with glutathione. Addition of HNE or the cell-permeable esters of glutathionyl-4-hydroxynonanol (GS-HNE) or glutathionyl-1,4-dihydroxynonanol (GS-DHN) to cultures of rat aortic smooth muscle cells stimulated protein kinase C, β1-integrin, and cell division, increased cell growth. The mitogenic effects of HNE, but not GS-HNE or GS-DHN, were abolished by glutathione depletion. Pharmacological inhibition or antisense ablation of aldose reductase (which catalyzes the reduction of GS-HNE to GS-DHN) prevented protein kinase C, NF-κB, and AP-1 stimulation and increased cell growth caused by HNE and GS-HNE, but not GS-DHN. The growth-stimulating effect of GS-DHN was enhanced in cells treated with antibodies directed against the glutathione conjugate transporters RILP76 (Ral-binding protein) or the multidrug resistance protein-2. Overexpression of RILP76 ablated the mitogenic effects of HNE and its glutathione conjugates, whereas ablation of RILP76 via RNA interference promoted the mitogenic effects. Collectively, our findings suggest that the mitogenic effects of HNE are mediated by its glutathione conjugate, which has to be reduced by aldose reductase to stimulate cell growth. These results raise the possibility that the glutathione conjugates of lipid peroxidation products are novel mediators of cell signaling and growth.

Incomplete reduction of oxygen leads to the generation of highly reactive species. When generated in high concentrations, the reactive oxygen species (ROS) cause tissue injury and cell death. Excessive ROS production has been linked to a number of degenerative diseases including atherosclerosis (1–3), Alzheimer disease (4, 5), and heart failure (6–8), as well as tissue injury and dysfunction associated with myocardial ischemia and reperfusion (9, 10) and diabetes (11, 12). Additionally, recent evidence suggests that ROS are physiological regulators and mediators of cell signaling due to cytokines and growth factors (13–16). Under physiological conditions, the biological effects of ROS are tightly regulated by chemical and enzymatic antioxidant defenses. However, when these defenses are overwhelmed by disease or injury, ROS attack many cell constituents, with unsaturated lipids being the main target. Unsaturated lipids provide a readily extractable proton to oxygen-free radicals. The resultant lipid radical is stabilized by the bis-allylic double bond system and rapidly accepts molecular oxygen to form peroxyl radicals. This initiates a series of complex, autocatalytic reactions that generate a variety of carbonyl compounds as their end products (17). Of these, aldehydes, such as 4-hydroxy-trans-2-nonalen (HNE) are the most abundant and hence of greater biological significance (18). Recently, it has been shown that lipid aldehydes such as malonaldehyde can also be formed by free radical attack to deoxyribose (19).

The formation of HNE and related aldehydes is symptomatic of oxidative stress, and these aldehydes or their protein adducts accumulate in diseased tissues such as atherosclerotic lesions (20–22), inflamed arteries (23), hypertrophic (24, 25) and ischemic hearts (26, 27), and the neuronal lesions associated with Parkinson (28) and Alzheimer (29) diseases. The accumulation of HNE and its protein adducts in diseased tissues indicates that lipid peroxidation products could contribute to disease pathology and progression. Indeed, in isolated cells, HNE activates several signaling pathways, including c-Jun NH2-terminal kinase (JNK) (30–34) and Nrf2-dependent induction of the scavenger receptor (35). Significantly, HNE is a potent vascular smooth muscle cell (VSMC) mitogen (36) and therefore could contribute to VSMC proliferation in atherosclerotic lesions. Nevertheless, the mechanisms by which HNE affects cell growth remain obscure.

Our previous studies show that VSMC transform HNE into multiple metabolites (37, 38). These include direct oxidation and reduction of HNE to 4-hydroxynonanoic acid and 1,4-dihydroxynonane (DHN), respectively. In addition, HNE also undergoes glutathione S-transferase (GST)-catalyzed conjugation to form GS-HNE, which is further reduced to GS-DHN by aldose reductase (AR; AKR1B4) (39–42). Both GS-HNE and GS-DHN can be actively extruded via membrane trans-

The abbreviations used are: ROS, reactive oxygen species; AR, aldose reductase; HNE, 4-hydroxy-trans-2-nonalen; GS-HNE-ester, glutathionyl-4-hydroxynonanol-ester; GS-DHN-ester, glutathionyl-1,4-dihydroxynonanol-ester; GS-ester, glutathione-mono-ethylster; MRP, multidrug resistance protein; VSMC, vascular smooth muscle cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; GST, glutathione-S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high performance liquid chromatography; PKC, protein kinase C; siRNA, small interfering RNA; FBS, fetal bovine serum; ESI/MS, electrospray ionization mass spectrometry; BSO, L-buthionine-(S,R)-sulfoximine.

* This work was supported in part by National Institutes of Health Grants GM71036 (to K. V. R.), DK36118 and EY01677 (to S. K. S.), HL59378 (to A. B.), and HL65618 (to S. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom the correspondence should be addressed. Tel.: 409-772-3926; Fax: 409-772-9679; E-mail: ssrivast@utmb.Du.

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port mechanisms. However, the functional significance of this metabolis-
mism is unclear and it is not known whether the mitogenic effects are
directly due to HNE or mediated by one of its metabolites. Therefore, we
tested the hypothesis that GS-DHN, the product of AR-catalyzed re-
duction of the glutathione conjugate of HNE, is the active metabolite
that mediates HNE signaling and mitogenesis. This view is consistent
with our previous observations that inhibition of AR (AKR1B4) pre-
vents protein kinase C activation and NF-κB activation (43–45) and
inhibits VSMC growth (37, 43). Our current results demonstrate the
selective ability of GS-DHN in mediating HNE-induced VSMC growth
and support the view that AR-catalyzed reduction of glutathione con-
jugates in general may be a critical and essential interface between
detoxification and signaling.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), phosphate-
buffered saline, penicillin/streptomycin solution, trypsin, and fetal bovine
serum (FBS) were purchased from Invitrogen. Consensus oligonucleo-
tide for NF-κB (5’-AGTTGAGGGGACTTTCCAGGC-3’) and AP-1
(5’-TTCCGGCTGACTCATCAAGCG-3’) transcription factors were
obtained from Promega Corp. The 3-(4,5-dimethylthiazol-2-yl)-2,5-di-
phenyl tetrazolium bromide (MTT), glutathione monoethyl ester, and
polyclonal antibodies against MRP-2 were obtained from Sigma. All
other reagents used were of analytical grade.

Cell Culture—Rat VSMC were isolated from healthy rat aorta and
characterized by smooth muscle cell-specific α-actin expression.
VSMC were maintained and grown in DMEM supplemented with 10% FBS
and 1% penicillin/streptomycin at 37 °C in a humidified atmo-
sphere of 5% CO₂.

Preparation of Cell Permeable GS-aldehyde Esters—HNE was synthe-
sized as described previously (41). The radiolabeled [4-3H]HNE was
synthesized from the dimethylacetal of HNE, which was oxidized to the
4-keto derivative using polymer-supported chromic acid as an oxidizing
agent. The resulting ketone was further reduced to the dimethylacetal of
HNE by using tritiated NaBH₄. The [4-3H]HNE obtained by acid
hydrolysis was purified on HPLC and stored in methylene chloride at
−20 °C until further use. The conjugate of glutathione-reduced ethyl ester
with HNE (GS-HNE-ester) was prepared by incubating 1 μmol of
[4-3H]HNE (55,000 cpm/nmol) with 5 μmol of GSH ethyl ester in 0.1 M
potassium phosphate, pH 7.0, for 1 h at room temperature. The reaction
was monitored by following the consumption of HNE at 224 nm. The
GS-HNE-ester conjugate was purified by reverse phase HPLC as described
below. For the synthesis of the reduced form of the esterified
glutathione-HNE conjugate (GS-DHN-ester), 100 nmoI of GS-HNE-
ester was incubated with 300 nmoI of NADPH and 100 μg of aldose
reductase in 0.1 M potassium phosphate, pH 6.0, for 3 h at 37 °C. The
reaction was monitored by following the consumption of NADPH at
340 nm. At the end of the incubation, the GS-DHN-ester conjugate
was separated from GS-HNE-ester by reverse phase HPLC as described
below.

HPLC Analysis—Synthesized standards and metabolites of GS-HNE
and GS-DHN-esters were separated by HPLC using a Varian reverse
phase ODS C₁₈ column pre-equilibrated with 0.1% aqueous trifluoro-
acetic acid. The compounds were eluted using a gradient consisting of
solvent A (0.1% aqueous trifluoroacetic acid) and solvent B (100% ace-
tonitrile) at a flow rate of 1 ml/min. The gradient was established such
that solvent B reached 24% in 20 min, 26% in 30 min, and was held at this
value for 10 min. Furthermore, in the next 10 min solvent B reached
60%, and in an additional 5 min it reached 100% and was held at this
value for 10 min.

Electrospray Ionization Mass Spectrometry—Chemical identities of
the GS-HNE and GS-DHN-esters were established by electrospray ion-
ization mass spectrometry (ESI/MS). The samples were analyzed on a
single quadrupole Micromass LCTZ instrument as described before (40).
The ESI⁺/MS operating parameters were as follows: capillary voltage, 3.0 kV;
capillary temperature, 13 V; extractor voltage, 9 V; source block temper-
ature, 100 °C; and dissolution temperature, 200 °C. Nitrogen at 3 psi
was used as nebulizer gas. Samples were reconstituted in 50 μl of ace-
onitrile/water/acetic acid (50/50/0.1) (v/v/v), and applied to the mass
spectrophotometer using a Harvard syringe pump at a rate of 5 μl/min.
Spectra were acquired at the rate of 200 atomic mass units/s over the
range of 20–2000 atomic mass units.

Metabolism of GS-HNE and GS-DHN-esters in VSMC—The growth
arrested rat VSMC (2 × 10⁶/well in six-well plates) were incubated with
radiolabeled GS-[3H]HNE and GS-[3H]DHN esters (1 μM; ~5,100
cpm) for 0, 30, 60, 120 min, and 24 h in a humidified CO₂ incubator.
The culture media were separated, filtered by using Amicon Centriprep
3-kDa membrane, and subjected to HPLC analysis as described above.

Cell Growth Studies—The rat VSMC were grown in DMEM and
harvested by trypsinization and plated in a 96-well plate at a density of
5,000 cells/well. Cells were grown for 24 h in the indicated media and
growth-arrested at 60–80% confluency for 24 h in media containing
0.1% FBS. Low serum levels were maintained during growth arrest to
prevent slow apoptosis that accompanies complete serum deprivation
of these cells. The growth-arrested cells were treated with (0.5-μM each
of) HNE, GS-HNE-ester, and GS-DHN-ester, in the absence and pres-
ence of AR inhibitors, sorbinil or tolrestat (10 μM each). The rate of cell
proliferation or apoptosis was determined by cell counts and MTT
assay. To examine the role of RLP76 (76-kDa Ral-binding, Rho/Rac-
GAP, and Rac effector protein) and MRP-2 (multidrug resistance-assoc-
ated protein-2) in mediating cell growth, growth-arrested VSMC were
washed with peptide-specific antibodies raised against RLP76 or
MRP-2 for 1 h followed by incubation with GS-DHN-ester (0–5 μM)
and the rates of cell proliferation or apoptosis were determined as
described above.

To study the effect of glutathione depletion, VSMC grown in DMEM
containing 10% FBS were treated with or without 25 μM BSO for 12 h.
The media was then replaced with fresh DMEM containing 0.1% FBS.
The cells were continuously cultured in the 0.1% FBS media without or
with BSO in the absence or presence of HNE (1 μM), GS-HNE-ester
(0.75 μM), or GS-DHN-ester (0.75 μM) for another 24 h. The rate of cell
proliferation or apoptosis was determined by cell count and MTT
assay.

Antisense Ablation of AR—Antisense ablation of AR (AKR1B4) was
carried out as described (43). Briefly, VSMC were transfected with 1 μM
AR antisense and mismatch control oligonucleotides in Opti-MEM for
12 h using Lipofectamine Plus (15 μg/mL) for a transfection reagent as
described by the supplier’s instructions. After 12 h, the medium was
replaced with DMEM (10% FBS). The cells were grown in this medium
for another 12 h and were then incubated with low serum DMEM (0.1% FBS)
for 24 h for serum starvation.

Overexpression and RNA Interference Ablation of RLP76—The
VSMC were transiently transfected with pcDNA3.1 vector contain-
ing RLP76 cDNA, or with the vector alone, using a Lipofectamine
Plus reagent as per supplier’s instructions (Invitrogen). Overexpres-
sion of RLP76 was monitored by Western blot analysis, using peptide-
specific RLP76 antibodies. To ablate RLP76, siRNAs were designed
to target the coding sequence of RLP76. The target sequences
(AAGAAAAAGCCAATTCAGGAGCC corresponding to nucleo-
tide 508–528 of RLP76 starting from AUG codon in the open read-
ing frame) were directed to the single-strand region according to the

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predicted secondary RNA structure. Sequences of the form (AA/CA)N19 with GC content <55% were selected from this region. Control non-silencing siRNA (fluorescein) was obtained from Qiagen. VSMC grown in DMEM containing 10% FBS and 1% penicillin and streptomycin at 37 °C and 5% CO2 were seeded on 6- or 96-well plates. When the cells reached 60–70% confluence (in 24 h), the media was replaced with fresh DMEM without serum, and the cells were incubated with siRNA to a final concentration of 100 nMol/liter and purified by HPLC with retention times of 28 and 31 min, respectively (Fig. 1A). ESI+/MS of HPLC peak I shows a molecular ion with an m/z value of 494.2, representing [M + H]+ of GS-DHN-ester (B), and ESI+/MS of peak II shows molecular ions with m/z values of 492.2 and 274.2 corresponding to [M + H]+ of GS-HNE-ester and its dehydrated daughter ion, respectively (C).

**Electrophoretic Mobility Gel Shift Assays—**Cytosolic and nuclear extracts were prepared as described (43). Consensus oligonucleotides for NF-κB and AP-1 transcription factors were 5′-ended labeled using T4 polynucleotide kinase. The assay procedure was as described before (43). Briefly, nuclear extracts prepared from various control and treated cells were incubated with the labeled oligonucleotides for NF-κB or AP-1 for 15 min at 37 °C, and the DNA-protein complex formed was resolved on 6.5% native polyacrylamide gels. The specificity of binding was examined by competition with an excess of unlabeled oligonucleotide. Supershift assay was also performed to determine the specificity of NF-κB binding to its specific consensus sequence by using anti-p65 antibodies. After electrophoresis, the gels were dried by using a vacuum gel dryer and autoradiographed on Kodak x-ray films. The radiolabeled bands were quantified by an Alpha Imager 2000 Scanning Densitometer equipped with AlphaEase™ version 3.3b software.

**Measurement of PKC—**The PKC activity was measured by using the Promega-Signa TECT™ total PKC assay system according to the manufacturer’s instructions. Briefly, aliquots of the reaction (25 mM Tris-HCl, pH 7.5, 1.6 mg/ml phosphatidylserine, 0.16 mg/ml dicylglycerol, and 50 mM MgCl2) were mixed with [γ-32P]ATP (3,000 Ci/mmol, 10 μCi/μl) and incubated at 30 °C for 10 min. To stop the reaction, 7.5 mM guanidine hydrochloride was added and the phosphorylated peptide was separated on binding paper. The extent of phosphorylation was detected by measuring radioactivity retained on the paper.

**Statistical Analysis—**Data are presented as mean ± S.D. and the p values were determined using the unpaired Student’s t test.

**RESULTS**

**Purification and Characterization of GS-HNE and GS-DHN-esters—**Because glutathione conjugates do not readily traverse cell membranes, to facilitate their entry GS-HNE and GS-DHN-esters were synthesized and purified by HPLC with retention times of 28 and 31 min, respectively (Fig. 1A). ESI+/MS of HPLC peak I (Fig. 1B) showed a strong molecular ion with an m/z value of 494.2, consistent with GS-DHN-ester, and that of peak II showed molecular ions with m/z values of 492.2 and 274.2 representing GS-HNE-ester and its dehydrated daughter ion, respectively (Fig. 1C).

**Metabolism of GS-HNE and GS-DHN-esters in VSMC—**The metabolism of GS-HNE and GS-DHN-esters in VSMC was investigated by quantifying their metabolites in the culture media and cell extracts. The results indicate that at 120 min only ~30% of GS-DHN-ester added was de-esterified in VSMC and transported out as only GS-DHN (Table 1). Furthermore, when GS-HNE-ester was added to the VSMC, 69% recovered as the GS-HNE-ester and 25% as free GS-HNE in the media. Only 7% radioactivity was eluted at the peak that corresponded to the elution time for DHN/4-hydroxynonanoic acid. No significant amounts (>2%) of the radiolabeled GS-HNE-ester or GS-DHN-ester and their metabolites were found in cell extracts. However, in the culture media, we have observed a time-dependent increase (30, 60, and 120 min, and 24 h) in the GS-HNE and GS-DHN levels and a time-dependent decrease in GS-HNE- and GS-DHN-esters (Table 1). These results suggest that GS-DHN does not dissociate, whereas some dissociation of GS-HNE could occur in VSMC.

**Mitogenic Effects of HNE and Its Metabolites in VSMC—**To examine changes in cell growth, early passage, serum-starved rat VSMC were incubated with either HNE or its glutathione conjugates: GS-HNE and GS-DHN. In agreement with our previous observations (36), we found
that incubation with HNE increased cell growth. A similar increase in growth was observed when the cells were incubated with GS-HNE- and GS-DHN-esters. As shown in Fig. 2, all three reagents, HNE, GS-HNE, and GS-DHN, increased cell proliferation at low concentrations, as determined by counting the number of cells and the MTT assay. With HNE, maximal stimulation of cell growth was evident at a concentration of 1 μM. Similarly, the glutathione conjugates stimulated cell growth at a maximal concentration of 0.75 μM, indicating that conjugation with glutathione does not abolish the mitogenic effects of HNE and that the glutathione conjugate of HNE whether untransformed or reduced is as potent a mitogen as the parent aldehyde.

Incubation of the cells with HNE or its glutathione conjugates at concentrations >1 μM led to a concentration-dependent increase in cell death. Significant loss of viability was observed when the cells were incubated with HNE or its conjugates at concentrations exceeding 5 μM. Such biphasic effects of HNE, with growth stimulation at low concentrations and cytotoxicity at high concentrations, have been reported before (36) and are consistent with the behavior of other oxidants such as hydrogen peroxide (46). Interestingly, high concentrations of the glutathione conjugates were also cytotoxic and both GS-HNE and GS-DHN at concentrations exceeding 5 μM induced cell death (Fig. 2, A and B).

Similar effects of HNE and its glutathione conjugates on cell growth suggest that all three reagents (HNE, GS-HNE, and GS-DHN) are equally mitogenic. Alternatively, conjugation with glutathione may be an essential "activation" step such that the glutathione conjugate, but not HNE per se, is the proximal mitogen. To distinguish between these possibilities, we first examined whether depleting glutathione could affect HNE-induced cell growth. For this, the cells were pretreated with 25 μM BSO for 12 h. This led to a 70% decrease in the intracellular level of reduced glutathione (data not shown). Both untreated and BSO-treated cells were then incubated with 1 μM HNE or 0.75 μM glutathione conjugates. As before, in the absence of BSO, HNE as well as its glutathione conjugates increased VSMC proliferation, whereas in the presence of BSO, only GS-HNE and GS-DHN were able to stimulate growth. HNE alone was ineffective (Fig. 2C). These results are consistent with the view that conjugation with glutathione is essential for HNE to stimulate cell growth and that only the glutathione conjugates of HNE, but not HNE itself, are smooth muscle cell mitogens.

In VSMC, HNE is conjugated to glutathione to form GS-HNE and a portion of this conjugate is reduced to GS-DHN by AR (37, 38). To distinguish whether GS-HNE or GS-DHN is mitogenic, we examined how inhibition of AR would affect the mitogenicity of the conjugates. For this series of experiments, growth-arrested VSMC were treated with the AR inhibitor, tolrestat (10 μM), in the absence and presence of GS-ester (10 μM). HNE (1 μM), GS-HNE (0.75 μM), and GS-DHN (0.75 μM), and changes in cell growth were determined as before by cell count and MTT assay. As shown in Fig. 3A, cell growth was stimulated by HNE, GS-HNE, and GS-DHN. A small, but statistically insignificant increase was also observed with the glutathione ester alone. Inhibition of AR prevented the increase in cell growth caused by HNE or GS-HNE, but not that due to GS-DHN. Similar results were obtained when the expression of AR was ablated by antisense RNA (Fig. 3B). These results show that treatment of cells in which >90% of AR protein and activity were decreased due to treatment with AR antisense (data not shown) with HNE or GS-HNE did not stimulate cell growth. In contrast, cells treated with GS-DHN

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FIGURE 2. Stimulation of VSMC growth by HNE and its glutathione conjugates. Serum-starved VSMC were stimulated for 24 h with the indicated concentrations (0–10 μM) of HNE, GS-HNE-ester, or GS-DHN-ester and cell growth was determined by MTT assay (A) and counting the number of cells (B) excluding trypan blue. Bars represent mean ± S.D.; n = 4; * and # p < 0.001 versus untreated cells. A, VSMC grown in DMEM containing 10% FBS were treated with or without 25 μM H11006 and changes in cell numbers and MTT reduction were measured. In parallel experiments, the cells were treated with GS-HNE-ester (7), HNE (8), GS-DHN-ester (9), and GS-DHN-ester (10) in the presence of 10 μM tolrestat. B, cells were either left untreated or treated with the transfection reagent (TR), vector alone (CR), or AR antisense (AR) as described in the text. After treatment, the cells were serum-starved for 24 h and were either left untreated (none) or treated with GS-HNE-ester, HNE, GS-HNE, or GS-DHN as indicated. Cells without (A) or with (B) AR ablation were incubated without or with 10 μM tolrestat in the absence and presence of 1 μM HNE, 0.75 μM GS-HNE-ester, or GS-DHN-ester for 24 h. Data represent mean ± S.D.; n = 4; * p < 0.001 tolrestat treated versus non-treated cells or AR ablated cells versus control cells. U, untreated cells; TR, cells treated with the transfection reagent; CR, cells treated with mismatched control oligonucleotides; and AR, cells treated with oligonucleotides antisense to AR.

FIGURE 3. Role of AR in regulating the growth promoting effects of HNE and its glutathione conjugates. A, serum-starved VSMC were either left untreated (lanes left to right, 1) or treated with tolrestat alone (2), GS-ester (3), HNE (4), GS-HNE-ester (5), or GS-DHN-ester (6) and changes in cell numbers and MTT reduction were measured. In parallel experiments, the cells were treated with GS-HNE-ester (7), HNE (8), GS-DHN-ester (9), and GS-DHN-ester (10) in the presence of 10 μM tolrestat. B, cells were either left untreated or treated with the transfection reagent (TR), vector alone (CR), or AR antisense (AR) as described in the text. After treatment, the cells were serum-starved for 24 h and were either left untreated (none) or treated with GS-HNE-ester, HNE, GS-HNE, or GS-DHN as indicated. Cells without (A) or with (B) AR ablation were incubated without or with 10 μM tolrestat in the absence and presence of 1 μM HNE, 0.75 μM GS-HNE-ester, or GS-DHN-ester for 24 h. Data represent mean ± S.D.; n = 4; * p < 0.001 tolrestat treated versus non-treated cells or AR ablated cells versus control cells. U, untreated cells; TR, cells treated with the transfection reagent; CR, cells treated with mismatched control oligonucleotides; and AR, cells treated with oligonucleotides antisense to AR.
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DHN is a product of AR-catalyzed reduction of GS-HNE, we infer required for stimulation of cell growth by GS-DHN. Because GS-HNE-ester (0.75 μM), or GS-DHN-ester (0.75 μM) for 4 h. Equal amounts of nuclear extracts were subjected to electrophoretic mobility gel shift assays as described under “Experimental Procedures” using specific NF-κB and AP-1 oligonucleotide probes. Additionally, serum-starved cells were either left untreated or treated with 10 μM tolrestat (C) or antisense AR oligonucleotides (D) for 24 h and then stimulated with HNE (1 μM), GS-HNE-ester (0.75 μM), or GS-DHN-ester (0.75 μM) for 4 h. After incubation, the membrane extracts from each sample were prepared and the PKC activity associated with each of these fractions was measured as described in the text using the Promega SigmaTect total PKC assay system. Each data point represents mean ± S.D.; n = 4; *, p < 0.001 versus aldehyde alone treated cells. U, untreated cells; TR, cells treated with the transfection reagent; CR, cells treated with mismatched control oligonucleotides; AR, cells treated with oligonucleotides antisense to AR; PS, preimmune sera; Ab, antibodies.

continued to proliferate at significantly elevated rates despite their near lack of AR. Collectively, these results suggest that AR is essential for the mitogenic effects of HNE and GS-HNE, but it is not required for stimulation of cell growth by GS-DHN. Because GS-DHN is a product of AR-catalyzed reduction of GS-HNE, we infer that GS-DHN, and not GS-HNE or HNE, is a direct stimulator of cell growth and that GS-HNE becomes mitogenic only when it is reduced by AR. In the case of HNE, it appears that the aldehyde has to be conjugated with glutathione and then reduced by AR to stimulate cell growth.

Activation of PKC and NF-κB by HNE and Its Metabolites—Growth factors and cytokines enhance smooth muscle cell growth by stimulating a number of diverse signaling mechanisms that converge on PKC activation, which in turn regulates the activity of key transcription factors including NF-κB and AP-1. Our previous studies show that inhibition of AR abolishes PKC activation as well as NF-κB- and AP-1-dependent gene transcription (43–45, 47). Therefore, we examined the effect of HNE, GS-HNE, and GS-DHN on the activation of NF-κB, AP-1, and PKC in VSMC in the absence and the presence of the AR inhibitor, tolrestat (10 μM). As shown in Fig. 4, A and B, incubation with HNE, GS-HNE, and GS-DHN led to an increase in the activity of NF-κB as well as AP-1. At the concentrations tested, all three mitogens were equally effective in stimulating the activity of these transcription factors. However, even though pretreatment with tolrestat prevented the HNE and GS-HNE-induced NF-κB and AP-1 activation, tolrestat did not prevent NF-κB or AP-1 activation in cells treated with GS-DHN. The inability of tolrestat in inhibiting the effects of GS-DHN was also evident in measurements of PKC. As shown in Fig. 4, HNE, GS-HNE, and GS-DHN were equally potent at stimulating PKC translocation from the cytosol to the membrane. However, in cells pretreated with tolrestat, HNE- and GS-HNE-mediated stimulation of PKC was significantly blunted, although the effect of GS-DHN on PKC activity was undiminished (Fig. 4C). Similar results were obtained with cells in which AR was ablated by antisense mRNA. Whereas this procedure severely attenuated HNE- and GS-HNE-induced PKC stimulation, it did not alter the response to GS-DHN (Fig. 4D). Together, the results obtained by measuring PKC, NF-κB, and AP-1 activity are consistent with the notion that HNE affects signaling events only after it is metabolized to GS-DHN.

GS-Lipid Aldehyde Conjugate Levels Regulate VSMC Growth—Conjugation of HNE with glutathione is followed by reduction and both reduced and oxidized conjugates are actively extruded from cells (37, 38). Hence, one way to enhance the cellular effects of the conjugates is to increase their intracellular residence time by inhibiting their extrusion. Because previous studies suggest that glutathione conjugates of lipophilic compounds are extruded via RLIP76 (48, 49) and MRP2 (50, 51) and that their transport activities can be inhibited by respective antibodies. Therefore, we tested if the inhibition of these transporters by their antibodies would affect the mitogenic ability of GS-lipid aldehyde conjugates by increasing their bioavailability. To assess the role of RLIP76, we determined the effect of four different antibodies raised against peptides representing the 3 distinct sites (Ab2, Ab3, and Ab4) of RLIP76 located inside the cell and one located outside the cell (Ab1). As shown in Fig. 5A, only the antibody that recognizes the extracellular domain of RLIP76 (Ab1, 10 μg/ml) increased the proliferative effects of GS-DHN, whereas the antibodies that recognize only the intracellular domains (Ab2, Ab3, and Ab4) were ineffective. Treatment with the anti-RLIP Ab1 at a concentration of 0–20 μg/ml enhanced the proliferative effects of GS-DHN, whereas at higher concentrations (30 μg) of the antibody a decrease in cell viability was observed (Fig. 5B). We next examined how anti-RLIP76-Ab1 (20 μg/ml) antibodies affect the GS-DHN-induced VSMC growth. As shown in Fig. 5C, GS-DHN caused proliferation of VSMC in a concentration-dependent manner up to 0.75 μM and a further increase in concentration was inhibitory to VSMC growth. Addition of RLIP76-Ab1 antibodies (20 μg/ml) significantly increased GS-DHN-induced VSMC growth as compared with GS-DHN alone up to 0.75 μM, but at a concentration >1 μM the growth promoting effect of RLIP antibodies decreased. These results indicate that by inhibiting the transport of GS-DHN, its intracellular concentration as well as its duration of stay significantly increases leading to increased cytotoxicity.

To further validate the role of RLIP76, we examined the effects of genetically ablating or overexpressing the protein. Transient transfection of VSMC with RLIP76 cDNA in pcDNA3.1 vector increased the RLIP expression more than 10-fold versus the expression in cells transfected with the vector alone (Fig. 6A, inset). Whereas control cells demonstrated an increase in the cell growth when treated with HNE, GS-HNE, and GS-DHN, cells transfected with RLIP76 cDNA decreased the cell growth (Fig. 6, A and B) indicating that facilitating the extrusion of the conjugate (decreasing its residence time in cells) prevents their mitogenic effects. Transient transfection of VSMC with RLIP76 siRNA
for 48 h almost (>90%) ablated the expression of RLIP76 as compared with the expression of RLIP76 in cells transfected with the vector alone (Fig. 6C, inset). RLIP76-ablated cells demonstrated some increase in cell growth (Fig. 6, C and D) when treated with HNE, GS-HNE, and GS-DHN over the control cells, indicating that absence of RLIP76 decreases the extrusion of the conjugates and thereby promotes their mitogenic effects.

In addition to RPLIP, glutathione conjugates are also extruded by MRP2 (50, 51). Therefore, we tested whether inhibition of MRP2 would also enhance the mitogenic effects of GS-DHN and whether its effects with RLIP76 will be additive. The results shown in Fig. 7, A and B, suggest that anti-RLIP76 antibodies (Ab1) at a concentration of 2 and 5 μg/ml potentiated 30 and 45% of VSMC growth induced by GS-DHN, whereas anti-MRP2 antibodies at a concentration of 2 and 5 μg/ml potentiated 32 and 20% of VSMC growth. Furthermore, incubation of VSMC with combined anti-RLIP and anti-MRP2 antibodies at concentrations of 2 and 5 μg/ml, respectively, caused 60 and 93% protection, suggesting that these antibodies specifically block the transport of the GS-DHN and increased the cytotoxicity of GS-DHN in VSMC. Taken together, these observations indicate that increasing the intracellular concentration or the bioavailability of GS-DHN by inhibiting its extrusion increases cell growth.

**DISCUSSION**

The results of this study demonstrate for the first time the ability of glutathione conjugates to stimulate cell signaling. We find that the glutathione conjugates of HNE activate PKC and stimulate NF-κB and AP-1, regulating cell growth, differentiation, and death. Glutathiolation of electrophilic metabolites, including lipid peroxidation products such as HNE, is currently believed to aid detoxification of hydrophobic xenobiotics and metabolites by increasing their solubility in water and extrusion from cells. Indeed, our previous results show extensive glutathiolation of HNE in car-
Of the multiple cell constituents, HNE and other unsaturated aldehydes display the highest reactivity with thiols (18). HNE readily forms Michael adducts with glutathione, and the reaction is further accelerated by GSTs. The rGST8-8 (mGST4 or hGSTT5.4) isoform is particularly effective with HNE (56–58). This is generally considered a detoxification step, although glutathione conjugates of α,β-unsaturated aldehydes have been shown to be toxic. Glutathione conjugates of 2-trans-butenal, 2-trans-hexenal, and 2-trans,6-cis-nonaladiene induce DNA damage (59), whereas the glutathione conjugate of acrolein stimulates radical formation (60) and causes nephrotoxicity (61). Similarly, glutathione conjugates of polyphenolic compounds and their metabolites retain or exceed the toxicity of the parent xenobiotic (62) and glutathiolation has been shown to cause bioactivation of haloalkenes (63). Hence, even though glutathiolation aids detoxification and extrusion, the pathophysiological significance of this transformation is not clear. In this regard, our demonstration that the glutathione conjugates of HNE are mitogenic and that decreasing glutathiolation (by depleting glutathione) prevents the mitogenic effects of HNE, but not of its glutathione conjugates, suggests that glutathiolation is not merely a detoxification step but a bioactivating mechanism that endows mitogenic properties to HNE.

Our previous studies show that heart (41), smooth muscle (37, 38), endothelial cells (64), and erythrocytes (65) excrete two distinct forms of glutathione conjugates of HNE. These include the GS-HNE conjugate and its reduced form, GS-DHN. In most cases, GS-DHN represents 50% of the total conjugate. We have also found that treatment with AR inhibitors prevents the reduction of the conjugate (37, 41, 64, 65) and that in vitro recombinant AR displays high affinity for glutathione conjugates of unsaturated aldehydes (39, 40, 42). Indeed, for C3 to C9 aldehydes, glutathiolation increases the catalytic efficiency of the enzyme (39). The AR active site conforms to an efficient glutathione binding site and provides specific ionic interactions for conjugate binding (40). Collectively, these studies suggest that AR is a high efficiency catalyst for the reduction of aldehyde-glutathione conjugates and that reduction by AR is a significant in vivo fate of the conjugate at least in cardiovascular tissues. Nevertheless, the significance of this transformation remains to be established. Both reduced and non-reduced forms of the conjugate are extruded and because conjugation with glutathione prevents HNE from participating in other Michael addition reactions, reduction might appear to be a superfluous or futile metabolism. However, the present study clearly suggests that reduction of the conjugate may be an essential step in modulating cell signaling and growth. This is supported by the observation that inhibition of AR prevents growth and PKC, NF-κB, and AP-1 activation by HNE and GS-HNE, but not that due to GS-DHN. Hence, reduction appears to be an essential transformation, which allows cells to “sense” HNE and trigger appropriate changes in cell signaling and growth.

To examine how increasing the cellular concentration of the conjugate alters the effects of the conjugate, we used antibodies against RLIP76 and MRP. Our previous studies show that in several cells glutathione conjugates of HNE are extruded by RLIP76 and that reconstitution of the RLIP76 protein in lipid vesicles increases the extrusion of glutathione conjugates (66, 67). In addition, MRP-2 has also been shown to extrude glutathione conjugates of a wide range of xenobiotics (50, 51). However, this is the first report indicating an important role of these transporters in removing glutathione conjugates from vascular smooth muscle cells. Because vascular injury during atherosclerosis and restenosis is associated with oxidative stress, it is likely that these transporters play an important role in regulating oxidative stress in the vessel wall. Moreover, given the...
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close structural similarity between glutathione conjugates and S-nitrosothiolglutathione, it is likely that these transporters may also be important in regulating the vascular effects of nitrosothiols. Further studies will be required to fully evaluate the significance of our finding that both MRP-2 and RPLIP76 mediate the efflux of glutathione conjugates in vascular smooth muscle cells.

Our observation that the mitogenic and the signaling effects of HNE are mediated by its glutathione conjugates raises the interesting possibility that cell growth, particularly under conditions of high oxidative stress, may be driven in part by glutathione conjugates of endogenous electrophiles. The levels of glutathione are a critical determinant of cell growth and differentiation and proliferating cells contain high levels of glutathione (15, 68, 69) and rapidly growing tumor cells express high levels of enzymes involved in glutathione synthesis and conjugation (70, 71). Because up-regulation of glutathione metabolism appears to be an integral part of abnormal cell growth and resistance to apoptosis, it is tempting to speculate that cell signaling due to glutathione conjugates may be a critical element in promoting cell survival and tumor growth. Hence inhibition of the glutathione conjugation or reduction may be a useful strategy for preventing abnormal growth during tumor development and atherogenesis.

In summary, the results of our study provide the first direct line of evidence that glutathione conjugates of lipid peroxidation products such as HNE are smooth muscle cell mitogens that stimulate PKC, NF-κB, and AP-1. Because this signaling cascade regulates cell growth as well as inflammation, its stimulation by glutathione conjugates could be significant in understanding how oxidative stress modulates cell function. Although the mechanism by which the conjugates stimulate cell signaling and growth remains unclear, our studies suggest that only the reduced form of the conjugate is effective, thereby supporting the view that AR-catalyzed reduction of the conjugate links metabolism and detoxification to cell signaling and growth. In addition, these observations also raise the interesting possibility that the previously documented involvement of AR in regulating signaling pathways and cell growth (43–45, 47) may be due to its ability to regulate the metabolism of endogenous conjugates. Additional work is required to address these issues.

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