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Oxidized linoleic acid regulates expression and shedding of syndecan-4

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Houston, Michelle, Matheau A. Julien, Sampath Parthasarathy, and Elliot L. Chaikof. Oxidized linoleic acid regulates expression and shedding of syndecan-4. *Am J Physiol Cell Physiol* 288: C458–C466, 2005. First published October 6, 2004; doi:10.1152/ajpcell.00001.2004.—Syndecan-4, a heparan sulfate proteoglycan that is widely expressed in the vascular wall and as a cell surface receptor, modulates events relevant to acute tissue repair, including cell migration and proliferation, cell-substrate interactions, and matrix remodeling. While syndecan-4 expression is regulated in response to acute vascular wall injury, its regulation under chronic proatherogenic conditions such as those characterized by prolonged exposure to oxidized lipids has not been defined. In this investigation, arterial smooth muscle cells were treated with 13-hydroperoxy-9,11-octadecadienoic acid (HPODE) and 13-hydroperoxy-10,12-octadecadienoic acid, oxidized products of linoleic acid, which is the major oxidizable fatty acid in LDL. Both oxidized fatty acids induced a dose-dependent, rapid upregulation of syndecan-4 mRNA expression that was not attenuated by cycloheximide. This response was inhibited by pretreatment with *N*-acetylcysteine, catalase, or MEK1/2 inhibitors, but not by curcumin or lactacystin, known inhibitors of NF- κ B. These data suggest that oxidized linoleic acid induces syndecan-4 mRNA expression through the initial generation of intracellular hydrogen peroxide with subsequent activation of the extracellular signal-regulated kinase signaling pathway via MEK1/2. Notably, the HPODE-induced enhancement of syndecan-4 mRNA was accompanied by accelerated shedding of syndecan-4. In principle, alterations in both the cell surface expression and shedding of syndecan-4 may augment a variety of proatherogenic events that occur in response to oxidized lipids.

heparan sulfate proteoglycan; smooth muscle cell

THE SYNDECANS ARE A FAMILY of transmembrane heparan and chondroitin sulfate proteoglycans involved in cell-matrix signaling (9, 40, 59). There are four members in mammals, syndecan-1 and -2 (fibroglycan), syndecan-3 (*N*-syndecan), and syndecan-4 (ryudocan) (3). Syndecans are found on all adherent cells, where, along with the glypicans, they serve as the primary source of cell surface heparan sulfate. They affect cell growth and attachment to the extracellular matrix (ECM) through covalently bound heparan sulfate chains, which act as coreceptors for growth factors such as bFGF and VEGF and bind to ECM components such as fibronectin and collagen (27, 54, 56). Syndecans also regulate lipid uptake through binding and internalization of lipoprotein lipase (LPL)-enriched low-density lipoprotein (LDL) particles (14).

Syndecan-4 has several features that are unique within the syndecan proteoglycan family. The ectodomain of syndecan-4 has a cell-binding site for an unknown cell surface ligand, thus enabling this proteoglycan to modulate cell-cell adhesion (31, 48). In addition, syndecan-4 also contains a protein kinase C (PKC) binding site on its cytoplasmic tail, and clustering of

syndecan-4 promotes PKC activation (38). Furthermore, syndecan-4 is found in focal adhesion sites, where it contributes to the structural and signaling functions of this macromolecular complex and may modulate cell-matrix interactions (10, 55). For example, increased expression of syndecan-4 may diminish cell motility (28).

Atherosclerosis is a chronic inflammatory disease marked by aberrations in cell migration, proliferation, and LDL internalization, among other factors (29). Changes in syndecan-4 expression are known to alter these cellular functions, but little is known about the regulation of syndecan-4 under proatherogenic conditions (14, 28, 54). Syndecan-4 is highly regulated in other inflammatory conditions such as ischemic myocardial injury, dermal wound repair, and arterial balloon injury (8, 15, 25). In this regard, we postulated that syndecan-4 expression would likely be regulated by factors that contribute to atherosclerotic lesion formation.

Considerable evidence has confirmed that prolonged exposure to oxidatively modified LDL as well as its lipid components can lead to atherosclerotic plaque formation. 13-Hydroperoxy-9,11-octadecadienoic acid (HPODE) and 13-hydroperoxy-10,12-octadecadienoic acid (HODE) are the oxidized products of linoleic acid, the major oxidizable fatty acid in LDL, and have been detected in oxidized LDL and atherosclerotic plaques (17, 46). HPODE is known to regulate the expression of catalase, ICAM, heme oxygenase-1, endothelial nitric oxide synthase, and perlecan and is a recognized smooth muscle cell (SMC) mitogen (1, 13, 33, 41–43). We report that expression of syndecan-4 mRNA and protein in vascular SMC (VSMC) is controlled by HPODE using a signaling pathway that is dependent on both intracellular hydrogen peroxide generation and the activation of p42/p44 MAPK. Furthermore, HPODE increases the amount of syndecan-4 ectodomains released into the medium. While not the focus of this report, it is conceivable that altered patterns of syndecan-4 expression and shedding contribute to proatherogenic cell behavior promoted by the presence of oxidized lipids within the arterial wall.

METHODS

Animals were handled in accordance with the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society.

Cell culture. Rat aortic SMC (rASM) from male Sprague-Dawley rats (4–6 wk old, 200 g) were isolated (53). Rats were anesthetized with ether, and their descending aortae were harvested. The adventitia was mechanically removed after treatment with 0.4 mg/ml collagenase, and the endothelium was scraped off the opened aorta. The medium was then minced and incubated with 0.7 mg/ml collagenase

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and 1 mg/ml elastase (Worthington Biochemical, Lakewood NJ). Cells were cultured in DMEM with 4.5 g/l glucose (GIBCO-BRL), 10% FBS (Hyclone Laboratories, Logan UT), 100 U/l penicillin, 100 µg/l streptomycin, and 10 U/l amphotericin B. SMC were characterized using immunostaining with SMC-specific myosin antibodies and SMC-specific actin antibodies (Sigma), and >95% of cells exhibited positive staining. Cells were then subcultured in DMEM with 1 g/l glucose, 10% FBS, 100 U/l penicillin, 100 µg/l streptomycin, and 10 U/l amphotericin B. Experiments were performed on cells from passages 6 and 7 that had been quiesced for 24 h in Ham's F-12 medium with 0.4% FBS. Although similar results were observed with DMEM, we preferred to use Ham's F-12 for our investigations because it contains lower amounts of antioxidants and higher amounts of metals.

Human aortic SMC (hASMC) were purchased from Clonetics and cultured in Clonetics SMC growth medium. Experiments were performed with cells from passages 8 and 9 that had been quiesced for 24 h in Clonetics smooth muscle basal medium (SmBM) medium with 0.4% FBS. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was obtained from Sigma (St. Louis, MO).

Preparation and measurement of HPODE. HPODE was prepared by oxidation of linoleic acid using lipoxygenase. Briefly, 0.4 mM linoleic acid in PBS was incubated with 700 U of soybean lipoxygenase at 37°C until the reaction was complete as monitored by diene absorbance at 235 nm. HPODE was then purified using ether extraction and resuspended in ethanol, and concentration was determined using 235-nm absorbance ($\epsilon = 2.52 \times 10^{-4} \text{ M}^{-1}$) or by performing a leucomethylene blue assay using commercial HPODE as a control. Leucomethylene blue reagent was prepared by dissolving benzoyl leucomethylene blue (TCI America, Portland, OR) in dimethylformamide to a concentration of 0.6 mg/ml, followed by the addition of 1.4% Triton X-100, 55 µg/ml hemoglobin, and 0.05 M K⁺-phosphate, pH 5, at a 1:12.5 dilution.

To determine the stability of HPODE in the presence of cells, Ham's F-12 medium with or without 60 µM HPODE was incubated at 37°C with confluent cells. At various times, aliquots of the medium were incubated with leucomethylene blue reagent for 15 min and measured at 660 nm. Within 1 h, the HPODE was completely consumed or reduced by cellular components. 13S-HODE was obtained from Sigma (St. Louis, MO).

Ribonuclease protection assay. Cells were lysed in 1 ml of TRIzol reagent (GIBCO-BRL), and RNA was extracted as recommended by the manufacturer. RNA probes radiolabeled with ³²P-UTP (Amersham) were synthesized using the Promega Riboprobe T7 system from pPMG/glyceraldehyde-4-phosphate dehydrogenase (GAPDH, rat and human; Pharmingen), pTRI-RNA-28S (Ambion), pcDNA3.1(+)/rAsyndecan-4, or PCR hAsyndecan-4. RNA samples (10 µg) were hybridized overnight at 45°C to respective probes using the Ambion RPAII kit, digested with RNase, and precipitated. RNA samples were then separated on a 5% polyacrylamide, 8 M urea, and Tris-borate-EDTA gel and exposed to film at -80°C. Levels of RNA were quantified by performing densitometry using IP Lab Spectrum Plus (Scanalytics, Fairfax, VA).

Western blotting. Polyclonal antibodies against the cytoplasmic (antibody 1423) and extracellular (antibody 1420) domains of rat syndecan-4 were a generous gift of Dr. Nicholas W. Shworak (Section of Cardiology, Department of Medicine, Dartmouth Medical School, Hanover, NH). A monoclonal antibody raised against human syndecan-4 (antibody 8G3) was a generous gift of Dr. Guido David (Laboratory for Glycobiology and Developmental Genetics, Department of Human Genetics, University of Leuven and Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium). All secondary antibodies were obtained from Jackson Laboratories (West Grove, PA).

Confluent cells were lysed in 20 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 10 mM EDTA, 1 mM PMSF, 10 mM N-ethylmaleimide, 1 µg/ml pepstatin A, and 0.5 µg/ml leupeptin at

4°C. Proteoglycans were then isolated on a DEAE-Sephadex column equilibrated with 20 mM Na⁺-acetate, pH 5, 200 mM NaCl, 6 M urea, 0.5% Triton X-100, 1 mM EDTA, and 1 mM Na⁺-sulfate, followed by elution with 20 mM Na⁺-acetate, pH 5, 1 M NaCl, 6 M urea, 0.5% Triton X-100, 1 mM EDTA, and 1 mM Na⁺-sulfate. Proteoglycans were precipitated by incubating the eluent with 4 volumes of 4°C ethanol and 2 nmol dextran sulfate for 2 h at 4°C, followed by centrifugation at 12,000 g for 1 h. The proteoglycans were resuspended in water, and the amount of protein was quantified using a Bradford assay (Bio-Rad) with albumin as a standard. Equal amounts of proteoglycan were then incubated overnight at 37°C with 1 U of heparitinase, 50 mU of chondroitinase ABC (Seikagaku America, Ijamsville, MD), 0.1 M Na⁺-acetate, pH 7, 0.1 mM Ca²⁺-acetate, 0.1% Tween 20, and 50 mM aminocaproic acid. The proteins were resolved using 12% SDS-PAGE and transferred to nitrocellulose. Syndecan-4 was detected using either antibody 1423/anti-rat IgG horseradish peroxidase (HRP) or antibody 8G3/anti-mouse IgG-HRP for rat and human SMC, respectively. Results were quantified using chemiluminescence and densitometry.

To determine the amount of syndecan-4 shed into the medium, conditioned medium was collected from confluent hASMC and centrifuged at 600 g for 5 min to remove any detached cells. Proteins from 100 µl of the supernatant were then bound to nitrocellulose paper using a slot blot apparatus (Bio-Rad). The nitrocellulose was blocked with 5% milk in 2% Tween 20-PBS and probed with the anti-human syndecan-4 (8G3) antibody, followed by an anti-mouse IgG-HRP. The amount of syndecan-4 was determined using chemiluminescence and densitometry.

DCF-DA fluorescence. Confluent rASM were incubated with 100 µM 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCF-DA mixed isomers; Molecular Probes, Eugene, OR) in Hanks' buffered salt solution at room temperature for 30 min, after which cells were washed with Hanks' buffered salt solution and exposed to oxidants for 10 min and then trypsinized for analysis by performing flow cytometry using FACScan (CellQuest software; Becton Dickinson). Standardized instrument settings were used for FL1-H fluorescence (350 V, amp gain 4.0) to detect DCF-DA.

Detection of phosphorylated ERK. Confluent rASM were treated with 20 µM HPODE for various times and then lysed with 20 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 10 mM EDTA, 0.1 mM PMSF, 0.5 µg/ml leupeptin, 10 µg/ml pepstatin A, and 5 µg/ml aprotinin A for 15 min at 4°C. Nonsoluble protein was removed by performing centrifugation at 16,000 g for 10 min at 4°C. Equal amounts of protein were then resolved using 12% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies to phosphorylated forms of p44 and p42 (Cell Signaling Technology, Beverly MA), followed by HRP-conjugated anti-mouse IgG antibodies. As a control, antibodies were removed with 62.5 mM Tris, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol, and then the membrane was reprobed with antibodies that recognize all forms of p44 and p42, followed by HRP-conjugated anti-rabbit IgG antibodies. Total and phosphorylated p44/p42 were quantified using chemiluminescence and densitometry.

Statistics. Data were analyzed using Student's *t*-test or one-way ANOVA to compare differences among average values obtained within groups. When ANOVA indicated a significant difference among groups as indicated by the *F*-statistic, comparison among means was performed using Tukey's *F*-test. Significance was accepted at *P* < 0.05. Unless otherwise noted, data are presented as means ± SE of at least four independent experiments.

RESULTS

HPODE induces syndecan-4 mRNA expression. To investigate the regulation of syndecan-4 under proatherogenic conditions, oxidized products of linoleic acid were incubated with arterial SMC. The oxidized free fatty acid HPODE caused a

dose-dependent increase in syndecan-4 mRNA (Fig. 1, A and B). The effect was transient, with a maximum response observed at 2–3 h. A lack of cell necrosis over the experimental dose range of HPODE was demonstrated using Trypan blue staining, although cell death was visibly evident at $>30 \mu\text{M}$ HPODE concentrations. Leucomethylene blue analysis, performed to quantify the amount of HPODE in the medium, revealed that HPODE was completely consumed or reduced within 1 h in the presence of cells.

Most preparations of HPODE contain at least some HODE, although leucomethylene blue analysis demonstrated that $>90\%$ of the oxidized lipid was HPODE at the time it was added to the cells. To determine whether HODE was the active element causing increased syndecan-4 mRNA expression, cells were exposed to purified 13S-HODE. While HODE did produce a dose-dependent increase in syndecan-4 mRNA, it was less pronounced than that associated with HPODE (Fig. 1C). Neither linoleic acid ($20 \mu\text{M}$) nor ethanol (the vehicle for HPODE) altered syndecan-4 mRNA expression (data not shown).

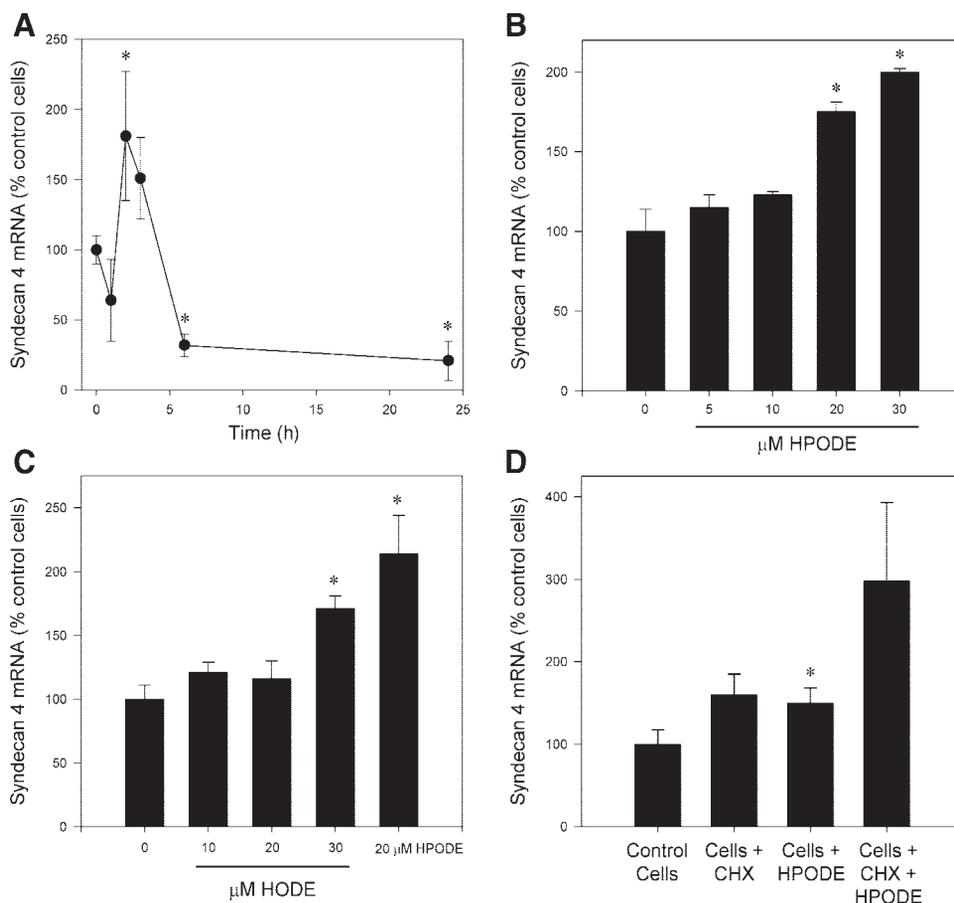
The effect of HPODE on syndecan-4 mRNA did not require de novo protein synthesis. Inhibition of protein synthesis before HPODE exposure by cyclohexamide did not attenuate enhanced syndecan-4 mRNA levels (Fig. 1D). Instead, cyclohexamide augmented the HPODE-dependent rise of syndecan-4 mRNA, a trait typical among early response genes and one that previously was demonstrated for syndecan-4 (8, 39, 52).

The observed increase in mRNA induced by HPODE could be attributed at least in part to stabilization of the syndecan-4

transcript. This was demonstrated by use of transcription inhibitor, DRB, which was added to the culture medium after exposure to HPODE (Fig. 2). A logarithmic fit of the data established that the half-life of syndecan-4 mRNA increased from 1.4 to 6.2 h after 2-h HPODE exposure ($P < 0.01$). However, after 18-h HPODE exposure, there was no difference between the stability of the syndecan-4 transcripts and that of control cells.

Generation of hydrogen peroxide is necessary but not sufficient for HPODE-mediated augmentation of syndecan-4 mRNA expression. HPODE generates intracellular oxidant stress and therefore may influence syndecan-4 expression through an oxidant-sensitive transcription factor. DCF-DA fluorescence confirmed that HPODE-treated cells were exposed to increased oxidative stress (Fig. 3A). Catalase, a scavenger of hydrogen peroxide, has been shown to protect cells against HPODE-induced apoptosis (45). Because catalase does not react directly with HPODE, these data suggest that HPODE stimulates the production of intracellular hydrogen peroxide, possibly by the peroxisome during HPODE degradation. Therefore, to determine the role of hydrogen peroxide production in HPODE signaling, cells were either 1) exposed to antioxidants such as catalase or *N*-acetylcysteine (NAC) to diminish hydrogen peroxide levels or 2) treated with aminotriazol, an inhibitor of intracellular catalase, to enhance hydrogen peroxide levels. Cells preloaded with catalase or NAC before exposure to HPODE did not exhibit an increase in syndecan-4 mRNA as large as that of cells treated with HPODE alone. Addition of aminotriazol produced a significant enhancement

Fig. 1. 13-Hydroperoxy-9,11-octadecadienoic acid (HPODE) and 13-hydroperoxy-10,12-octadecadienoic acid (HODE) regulate syndecan-4 mRNA levels. Rat arterial smooth muscle cells (rASMC) were exposed to $20 \mu\text{M}$ HPODE for various times (A) or to increasing amounts of HPODE (B) or HODE (C) for 3 h. D: rASMC were incubated with or without $10 \mu\text{M}$ cyclohexamide for 1 h, followed by addition of medium $\pm 20 \mu\text{M}$ 13S-HPODE for 3 h. Data are presented as percentages of untreated cell syndecan-4/GAPDH mRNA values. A higher dose of cyclohexamide ($20 \mu\text{M}$) also was used, with the same effect. $*P < 0.05$.



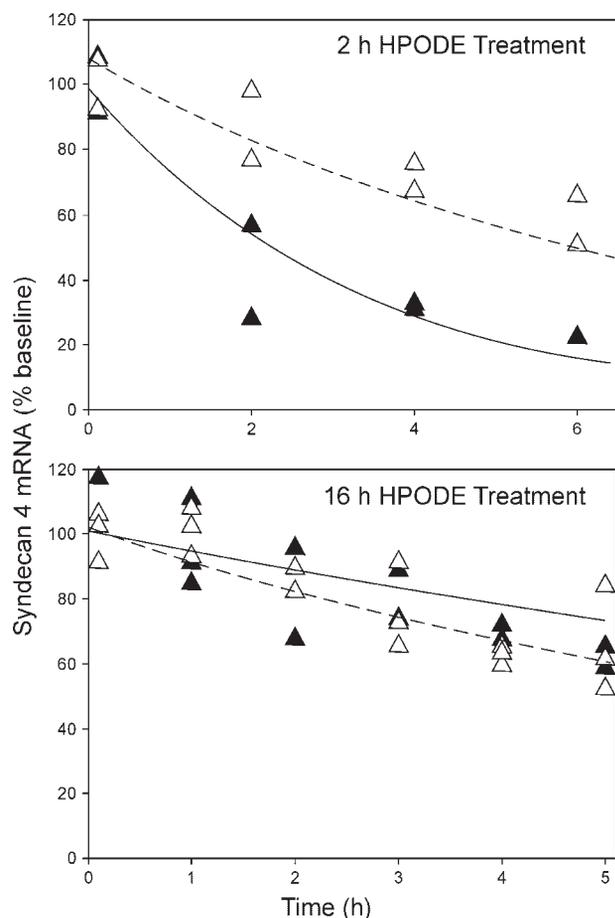


Fig. 2. HPODE-induced increase in mRNA could be attributed at least in part to stabilization of syndecan-4 mRNA. To determine the stability of the syndecan-4 transcript, rat vascular SMC were exposed to medium with (▲) or without (△) 20 μ M 13S-HPODE for 2 or 16 h, followed by addition of 100 μ M 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole to inhibit transcription. Total RNA was then harvested at various times. Syndecan-4 mRNA levels were determined using an RNase protection assay (RPA) and normalized using 28S RNA. Data are presented as percentages of baseline syndecan-4/28S RNA values.

of the HPODE effect (Fig. 3B). Furthermore, the addition of butyric acid, another compound shown to produce hydrogen peroxide when catabolized by the peroxisome, also increased syndecan-4 expression in VSMC. These results support the notion that intracellular production of hydrogen peroxide is a mediator of the HPODE-induced increase in syndecan-4 mRNA. Notably, the addition of extracellular hydrogen peroxide in the concentration range of 50–200 μ M, either as a bolus or produced in situ with glucose oxidase, failed to alter syndecan-4 mRNA levels (data not shown).

Because the syndecan-4 promoter contains binding sites for NF- κ B, which may be activated by hydrogen peroxide or oxidized LDL, it was anticipated that NF- κ B would be a mediator of HPODE signaling (18, 30, 51). To test this hypothesis, cells were treated with increasing doses of NF- κ B inhibitors curcumin and lactacystin before HPODE exposure. Both compounds failed to diminish the effect of HPODE on syndecan-4 (Fig. 4). Thus, unlike TNF- α stimulation of syndecan-4 expression, HPODE-induced enhancement of syndecan-4 mRNA does not appear to rely on activation of NF- κ B (58).

Activation of ERK is required for HPODE-mediated upregulation of syndecan-4 mRNA. Extracellular signal-regulated kinase (ERK) has been shown to mediate EGF-dependent shedding of syndecan-4 in immortalized endothelial and epithelial cells (12). To determine whether ERK activation was involved in the HPODE-dependent increase in syndecan-4 mRNA expression, ERK phosphorylation was examined using antibodies specific to the dual phosphorylated, active forms of p44 and p42 ERK. Upon addition of HPODE, ERK was phosphorylated in a time-dependent manner (Fig. 5A) that was abrogated by exposing the cells to catalase before HPODE exposure. Furthermore, treatment of cells with PD-98059 or U-0126, specific inhibitors of MEK1/2, prevented the increase in syndecan-4 mRNA after addition of HPODE (Fig. 5B). SB-203580, a specific inhibitor of p38 MAPK, was less effective. Addition of hydrogen peroxide alone caused ERK phosphorylation (data not shown) (18, 57), which suggests that HPODE activates the ERK signal transduction pathway through production of intracellular hydrogen peroxide. Thus HPODE-induced syndecan-4

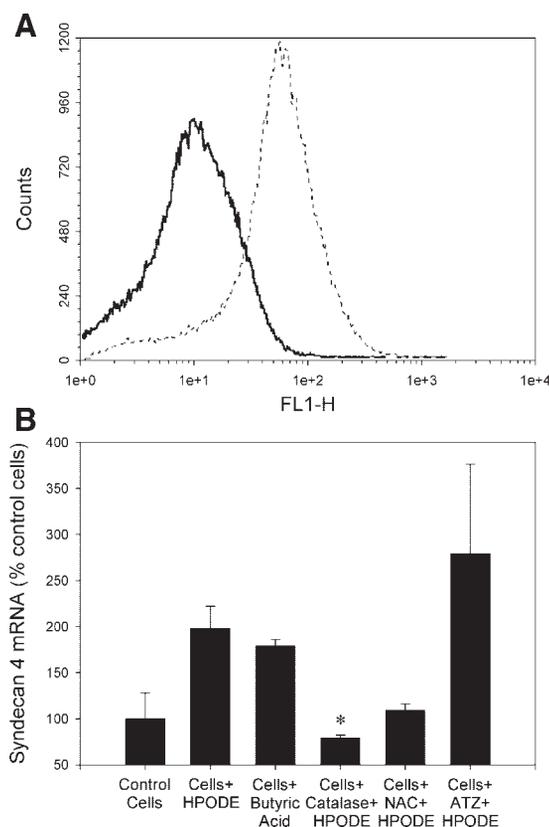
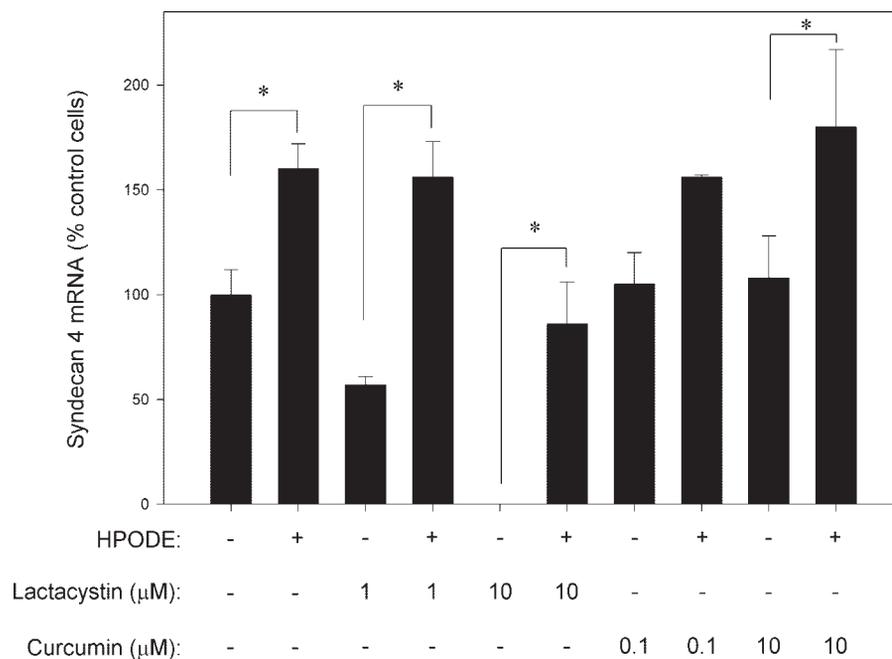


Fig. 3. HPODE regulation of syndecan-4 mRNA is dependent on intracellular hydrogen peroxide production. *A*: oxidative stress imposed on rat SMC before (solid line) and after (dashed line) exposure to 20 μ M 13S-HPODE for 30 min was visualized with 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) fluorescence and quantified with fluorescence-activated cell sorting analysis. *B*: to determine whether hydrogen peroxide mediates the 13S-HPODE-dependent increase in syndecan-4 mRNA, cells were incubated with or without 3,000 U/ml catalase or 30 mM *N*-acetylcysteine (NAC) for 2 h or 500 μ M aminotriazol (ATZ) for 1 h. The cells were then washed and exposed to medium \pm 20 μ M 13S-HPODE or 100 μ M butyric acid for 3 h, after which RNA was collected. Syndecan-4 and GAPDH mRNA were quantitated with RPA, and syndecan-4 mRNA levels were normalized with GAPDH levels. Data are presented as percentages of untreated cell syndecan-4/GAPDH mRNA values. * P < 0.05 compared with cells treated with HPODE alone.

Fig. 4. Curcumin and lactacystin do not inhibit the HPODE-dependent increase in syndecan-4 mRNA. Rat SMC were incubated with varying concentrations of curcumin or lactacystin for 2 h before addition of 20 μ M 13S-HPODE for 3 h. RNA was then collected and probed for syndecan-4 and GAPDH mRNA using RPA. Data are presented as percentages of untreated cell syndecan-4/GAPDH mRNA values. * $P < 0.05$ compared with corresponding non-HPODE-treated group.



mRNA expression appears to depend on intracellular hydrogen peroxide production that activates MEK1/2 to phosphorylate ERK.

HPODE signaling does not occur through PPAR. HPODE and HODE are known ligands for peroxisome proliferator-activated receptor- γ (PPAR- γ) in SMC (36, 47). To determine whether the syndecan-4 mRNA response to HPODE was partially mediated through PPAR- γ , cells were exposed to two PPAR- γ agonists over a concentration range that included the EC₅₀ values for PPAR- γ activation. Neither caused a significant increase in syndecan-4 mRNA, and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15dPGJ₂) actually produced a dramatic decrease in mRNA (Fig. 6). These results indicate that PPAR- γ receptors are not involved in the regulation of syndecan-4 mRNA by HPODE.

Syndecan-4 shedding is stimulated by HPODE. HPODE regulated syndecan-4 protein as well as mRNA. Cellular syndecan-4 protein was measured in both rat and human SMC using Western blot analysis of purified cellular proteoglycans. There was a marked decline in cellular syndecan-4 protein within 2 h after exposure to HPODE (Fig. 7, A and B), but 24 h after HPODE exposure, protein concentrations began to recover. Shed syndecan-4 was detected in the culture medium of hASMCM as early as 1 h after treatment with HPODE, which likely accounts for the diminished cellular syndecan-4 concentrations (Fig. 7C).

DISCUSSION

Syndecans are highly regulated during repair of cutaneous and arterial wounds, as well as in response to ischemic myocardial injury (8, 16, 25). In a myocardial infarction model, syndecan-4 expression is predominant in newly formed granulation tissue (25). Of note, delayed formation of granulation tissue and decreased angiogenesis have been observed after cutaneous injury in syndecan-4^{-/-} transgenic mice (11). In this report, we have demonstrated using rat and human VSMC that

syndecan-4 is also regulated by 13S-HPODE, an oxidized lipid present in oxidized LDL and atherosclerotic plaques. Although there is an initial enhancement of syndecan-4 mRNA expression upon HPODE exposure, due in part to the stabilization of syndecan-4 mRNA, a persistent depression of both syndecan-4 mRNA and protein was subsequently observed.

The transient increase of syndecan-4 mRNA after HPODE treatment is dependent on intracellular production of hydrogen peroxide, which activates ERK by MEK1/2. This was demonstrated by the ability of catalase and MEK1/2 inhibitors to prevent both HPODE-induced activation of ERK and enhanced expression of syndecan-4. However, the presence of hydrogen peroxide alone did not effect syndecan-4 mRNA expression, even though the addition of extracellular hydrogen peroxide activated ERK. Thus an additional factor or factors were required to mediate HPODE-induced alterations in syndecan-4 expression. NF- κ B was considered to be a candidate mediator of HPODE signaling, because an NF- κ B binding site exists on the syndecan-4 promoter and HPODE can activate NF- κ B (37, 51). Furthermore, Zhang et al. (58) reported that TNF- α stimulates syndecan-4 expression through NF- κ B. Despite these observations, two different inhibitors of NF- κ B activation failed to prevent an increase in syndecan-4 mRNA upon HPODE exposure. Other potential transcription factor binding sites on the syndecan-4 promoter include those for Sp1, activator protein (AP)-2, leader binding protein-1 H4TF-2, and E- α H-box (51). Of these, only AP-2 and Sp1 have been shown to be influenced by oxidative stress (2, 20). However, it is unlikely that AP-2 plays a significant role in this process, because Natarajan et al. (37) recently observed that AP-2 is not activated by HPODE in porcine VSMC. While HPODE's capacity to influence syndecan-4 by an Sp1-dependent pathway was not directly determined in our investigation, the response of syndecan-4 mRNA to 15dPGJ₂ suggests that a potential role for Sp1 may exist. Specifically, we observed that 15dPGJ₂, which inhibits Sp1 binding to DNA through the activation of

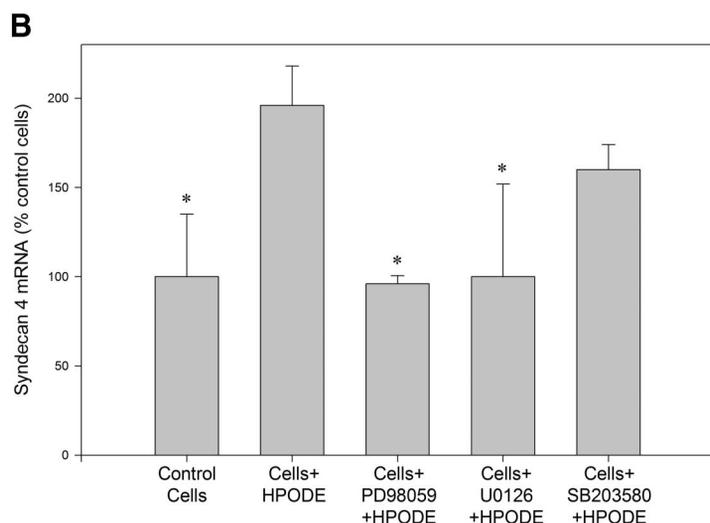
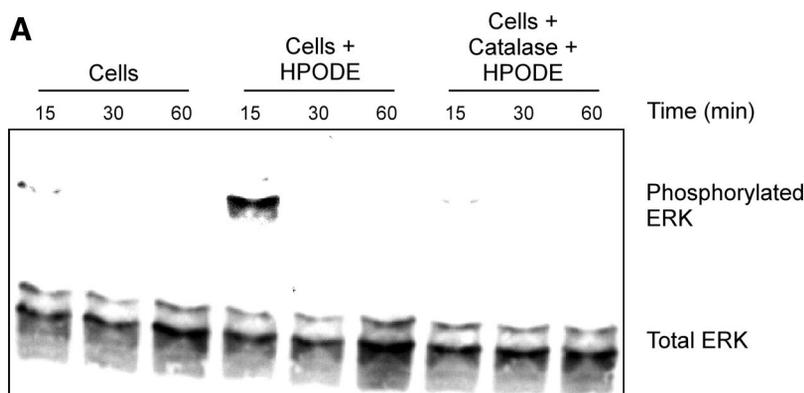


Fig. 5. HPODE regulation of syndecan-4 mRNA is dependent on extracellular signal-regulated kinase (ERK) activation. *A*: rASMC were incubated with or without 3,000 U/ml catalase, after which the cells were washed and exposed to 20 μ M 13S-HPODE for various times. Cell lysates were collected as described in METHODS and probed with antibodies for phosphorylated ERK and total ERK. *B*: rASMC were incubated for 1 h with 20 μ M PD-98059, 300 nM U-0126, or 10 μ M SB-203580 followed by 20 μ M 13S-HPODE for 3 h. RNA was then collected and probed for syndecan-4 and GAPDH mRNA using RPA. Data are presented as percentages of untreated cell syndecan-4/GAPDH mRNA values. * $P < 0.05$ compared with cells treated with HPODE alone.

PPAR- γ (50), reduced the expression of syndecan-4. Notably, recent studies have suggested that HPODE causes a decrease in PPAR- γ activity by MAPK-induced phosphorylation (19) and thereby could, in principle, potentiate Sp1 binding and perhaps, as a consequence, increased syndecan-4 expression. Nonetheless, 15dPGJ₂ has biological functions other than the activation of PPAR- γ , which may be responsible for this effect. For example, 15dPGJ₂ has been shown to augment intracellular glutathione levels, and thus it has an antioxidant effect similar to that of NAC. Although by itself NAC did not influence basal syndecan-4 levels, it did slightly inhibit the HPODE effect (Fig. 2B). Of greater significance, 15dPGJ₂ also inhibits ubiquitin isopeptidase and thereby interferes with the proteasome pathway (35). Indeed, we observed that lactacystin, which also inhibits the proteasome pathway, caused a dramatic decrease in syndecan-4 mRNA levels (Fig. 3). Therefore, further studies are needed to establish which supplementary factors, in addition to intracellular production of hydrogen peroxide and ERK activation, are involved in syndecan-4 regulation by HPODE.

It is unusual that the addition of extracellular hydrogen peroxide did not produce an increase in syndecan-4 mRNA, despite the reliance of syndecan-4 regulation on intracellular hydrogen peroxide production. Nonetheless, similar phenomena have been observed in other studies. For example, TNF- α stimulation of NF- κ B in dermal fibroblasts was enhanced by addition of the catalase inhibitor aminotriazol, but NF- κ B

activation was not observed upon the addition of extracellular hydrogen peroxide (23). Therefore, it is possible that oxidant-dependent pathway signaling requires the generation of high concentrations of hydrogen peroxide within a specific subcel-

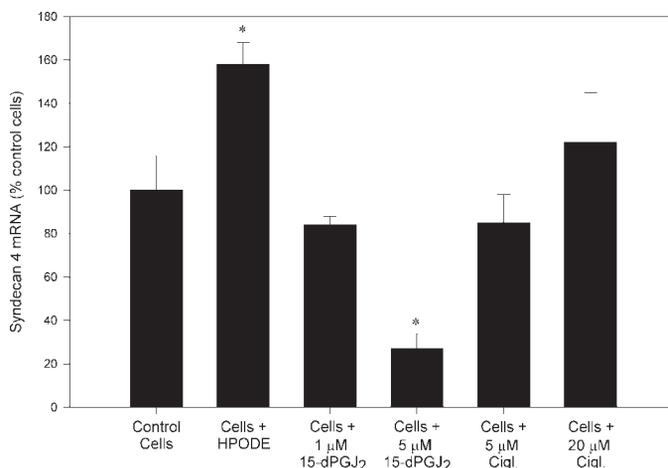


Fig. 6. Peroxisome proliferator-activated receptor (PPAR) ligands do not induce syndecan-4 mRNA expression. rASMC were treated with 20 μ M 13S-HPODE, 15-deoxy- Δ -prostaglandin J₂ (15dPGJ₂), or ciglitazone (Cigl.) for 3 h. RNA was then collected and probed for syndecan-4 and GAPDH mRNA using RPA. Data are presented as percentages of untreated cell syndecan-4/GAPDH mRNA values. * $P < 0.05$ vs. control cells.

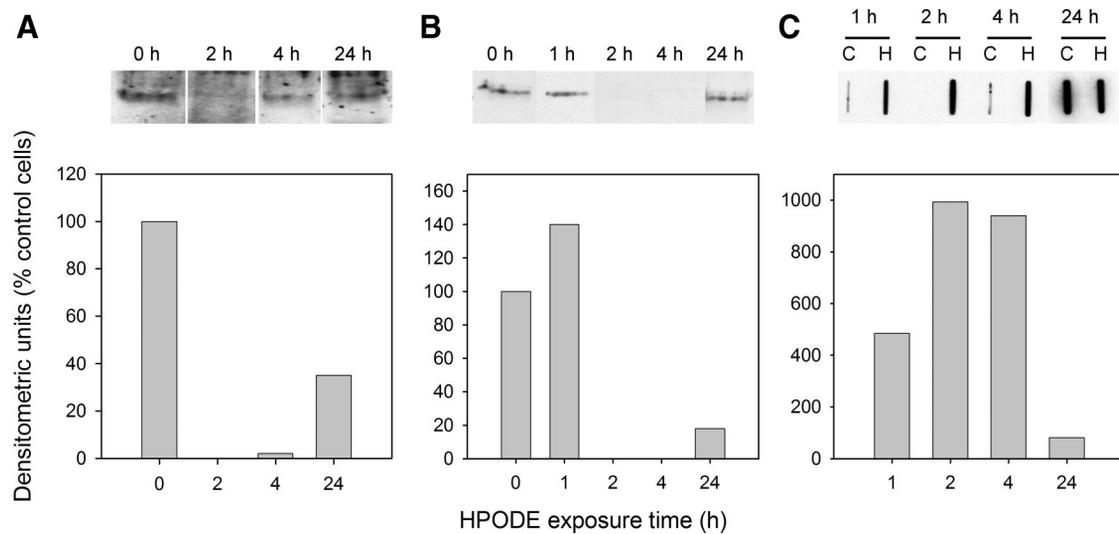


Fig. 7. Syndecan-4 protein is downregulated by HPODE as a result of shedding. Rat (A) or human SMC (B, C) were exposed to 20 or 10 μ M 13S-HPODE, respectively, for 1–24 h. Cellular syndecan-4 was detected on immunoblots after partial purification of cellular proteoglycans on DEAE columns. Shed syndecan-4 was collected from control cells (C) or HPODE-treated cells (H) at various times and applied to nitrocellulose blots for slot blot analysis. Syndecan-4 was measured with antisera raised against extracellular domain of rat syndecan-4 or with monoclonal IgG against human syndecan-4 followed by ECL detection.

lular compartment that cannot easily be achieved by the addition of hydrogen peroxide to cell medium.

The diminished cellular levels of syndecan-4 protein observed after the exposure of SMC to HPODE were due at least in part to loss of syndecan-4 into the medium, a process termed shedding. Shed syndecan-4 was seen as early as 1 h after the addition of HPODE, before any changes in mRNA expression. Shedding of syndecan-4 is also reported to occur with EGF or thrombin receptor activation, plasmin, heat shock, and osmotic stress and is known to occur *in vivo*, where the extracellular domain of syndecan-4 is found in dermal wound fluid (12, 49). Once shed, soluble syndecan-4 ectodomains may exert a significant biological effect, mainly through the heparan sulfate glycosaminoglycans attached to the core protein. Kainulainen et al. (21), for instance, demonstrated that soluble heparan sulfates on syndecan-1 and -4 bind to a variety of proteases and prevent their inactivation by protease inhibitors. Heparan sulfates also can sequester within the ECM both growth factors and proinflammatory chemokines, such as RANTES, monocyte chemoattractant protein-1, and IL-8 (24). In the process, these factors are likely protected from heat-, pH-, and protease-related degradation mechanisms, and their ability to interact with cell bound receptors is probably limited until release by heparanase-induced heparan sulfate cleavage (22). Shedding of the syndecan-4 ectodomain has not been studied in response to bFGF, PDGF, and TNF- α ; compounds that are known to regulate syndecan-4 mRNA expression. Therefore, HPODE is the only compound that is known to date to regulate both syndecan-4 mRNA expression and ectodomain shedding.

It is also interesting to speculate that direct reduction in cell surface syndecan-4 levels produced by HPODE, in addition to syndecan shedding, may provide a second mechanism for altering cell behavior. As a case in point, changes in cell surface syndecans have been shown to influence both cell proliferation and internalization of lipids. An immortalized endothelial cell line overexpressing syndecan-4 but not syndecan-1 or glypican-1 demonstrated enhanced bFGF-stimulated

growth and migration (54). Similarly, expression of syndecan-1 in Chinese hamster ovary (CHO) cells enhanced the association and internalization of LDL enriched with LPL (14). Syndecan-4 also modulates cellular motility; hence, a loss of syndecan-4 from SMC may influence the migration of these cells into the neointima. For example, overexpression of syndecan-4 inhibits motility in CHO cells, and ARH-77 B lymphoid cells transfected with syndecan-4 failed to invade collagen gels (27, 28). Of interest, Echtermeyer et al. (11) observed that syndecan-4^{-/-} fibroblasts displayed reduced motility. This seemingly conflicting observation emphasizes that the capacity of syndecan-4 to enhance or inhibit cell motility is context dependent. Other factors such as the characteristics of associated chemotactic and haptotactic gradients influence adhesion processes modulated by syndecan-4 (6, 7, 44).

Although additional investigations are required to determine the physiological significance of HPODE-induced changes in syndecan-4 expression and shedding, in principle this phenomenon might augment a variety of proatherogenic events that occur in response to oxidized lipids (4, 5, 34). The release of syndecan ectodomains into the ECM of the vascular wall may well lead to an increase in protease activity and the proliferation of SMC as well as assist in establishing chemotactic gradients for the recruitment of myofibroblasts, SMC, and leukocytes (21, 22, 24, 26). Moreover, SMC release of heparan sulfate-containing syndecan ectodomains could potentially increase the local retention of LPL-enriched LDL. It is conceivable that as a consequence of this effect, a positive feedback loop is created whereby further oxidation of LDL is promoted that could lead to foam cell formation as well as persistent changes in syndecan-4 expression (14, 32).

GRANTS

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