Inflammatory Cytokines and Fatty Acids Regulate Endothelial Cell Heparanase Expression†

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ABSTRACT: Heparan sulfates, the carbohydrate chains of heparan sulfate proteoglycans, play an important role in basement membrane organization and endothelial barrier function. We explored whether endothelial cells secrete a heparan sulfate degrading heparanase under inflammatory conditions and what pathways were responsible for heparanase expression. Heparanase mRNA and protein by Western blot were induced when cultured endothelial cells were treated with cytokines, oxidized low-density lipoprotein (LDL) or fatty acids. Heparanase protein in the cell media was induced 2−10-fold when cells were treated with tumor necrosis factor α (TNFα) or interleukin 1β (IL-1β). Vascular endothelial growth factor (VEGF), in contrast, decreased heparanase secretion. Inhibitors to nuclear factor-κB (NFκB), PI3-kinase, MAP kinase, or c-jun kinase (JNK) did not affect TNFα-induced heparanase secretion. Interestingly, inhibition of caspase-8 completely abolished heparanase secretion induced by TNFα. Fatty acids also induced heparanase, and this required an Sp1 site in the heparanase promoter. Immunohistochemical analyses of cross sections of aorta showed intense staining for heparanase in the endothelium of apoE-null mice but not wild-type mice. Thus, heparanase is an inducible inflammatory gene product that may play an important role in vascular biology.

Mammalian heparanase was first cloned in 1999 (*1*−*3*); however, investigation of its regulation is limited. The human heparanase enzyme is expressed as a 543 amino acid precursor protein that is modified by proteolytic cleavage to remove the first 157 amino acids to generate the mature active 50 kDa enzyme (*1*). Heparanase specifically cleaves heparan sulfate (HS)[1] glycosaminoglycans from HS proteoglycan (HSPG) core proteins and releases sequestered bioactive molecules. Locally produced heparanase may facilitate basement membrane degradation and allow migration of cells (*4*). Intracellular heparanase activity has been demonstrated in many cells including neutrophils, platelets, T-cells, and tumor cells (*4*).

Despite the important roles heparanase may play in inflammation, little is known about the regulation of this enzyme. Although endothelial cells have been postulated to produce heparanase in situations such as wound healing and angiogenesis, such activities have not been characterized. We previously showed that endothelial cells secrete a HS degrading activity when stimulated with the atherogenic agents oxidized low-density lipoprotein (Ox-LDL) and lysolecithin (*5*, *6*). However, it was unclear whether this HS degrading activity is related to altered heparanase gene expression.

Here we provide evidence that endothelial cells express the mammalian heparanase. Heparanase expression is increased by a variety of inflammatory cytokines and downregulated by antiinflammatory agents. Fatty acids (FA) also increase heparanase expression. Finally, we demonstrate elevated heparanase levels in atherosclerosis-prone arteries.

MATERIALS AND METHODS

Cell Culture. Human microvascular endothelial cells (HMVEC) (Clonetics) were cultured according to manufacturer's instructions. Bovine aortic endothelial cells (BAEC) and human kidney HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin and streptomycin. Human monocyte THP1 cells were maintained in RPMI 1640 with 10% FCS and antibiotics. In our previous study, we showed heparanase induction by Ox-LDL in bovine endothelial cells (*5*); in this study, we extended our work to human endothelial cells as well.

Reagents and Treatments. Tumor necrosis factor α (TNFα, R&D Systems), interleukin 1β (IL-1β, R&D Systems), vascular endothelial growth factor (VEGF, Chemicon International), dexamesathone (Sigma), PDTC (Ammonium pyrrolidine dithiocarbamate (Sigma P8765)), SN50 (a peptide, no specific define) (Biomol Research Laboratories), PI3-kinase inhibitors wortmannin and LY294002 (Calbiochem),

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[1] Abbreviations: HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; LpL, lipoprotein lipase; HMVEC, human microvascular endothelial cells; BAEC, bovine aortic endothelial cells; Ox-LDL, oxidized low-density lipoprotein; EMSA, electrophoretic mobility shift assay; FA, fatty acid; PPAR, peroxisome proliferator-activated receptor.

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MAP kinase (MAPK)/c-jun kinase (JNK) inhibitors (Calbiochem), and caspase inhibitor (Calbiochem) were purchased commercially. Ox-LDL was prepared by incubating LDL ($d = 1.019-1.063$ g/mL) with 10 μM copper chloride for 24 h, and the amount of oxidation was assessed by measuring thiobarbituric acid reactive substances as described (*7*). The thiobarbituric acid reactive substance values of Ox-LDL were 16−20 nmol of malondialdehyde equivalents/mg of protein. Thus the Ox-LDL used was moderately oxidized (compared to extensively oxidized LDL, which typically has values of 40−45 nmol of malondialdehyde).

Oleic acid and FA-free bovine serum albumin (BSA) (cat. no. A6003) were purchased from Sigma. Oleic acid−BSA complex (5:1) was made as follows: 1 g of oleic acid was first dissolved in 500 μL of ethanol, mixed with 4 mL of 30% $NaHCO_3$, and then added to 216 mL of 20% BSA. The solution was then adjusted to pH 7.4 with NaOH and stored in aliquots at −20 °C. Troglitazone and ciglitazone were from BioMol Research Inc. (Plymouth Meeting, PA). Mithramycin was obtained from Sigma. [^{35}S]-Sulfate and [^{32}P]-dCTP were purchased from Amersham. Cells, >90% confluent, were treated with various reagents for the indicated times in media containing 1% serum.

Northern Blot. Ten micrograms of RNA was used for RNA electrophoresis. A 1.6 kb fragment of human heparanase cDNA used as a probe was labeled with [^{32}P]-dCTP by the Megaprime DNA labeling system (Amersham). The membrane was hybridized with Rapid-hyb buffer (Amersham). After hybridization, the same membrane was stripped and rehybridized using a GAPDH probe as internal control.

Western Blot. Polyclonal antibodies to heparanase were raised in rabbit against a 15 amino acid peptide representing amino acids 437−451 (CTNTDNPRYKEGDLT) of the human heparanase (*3*). The antibody was affinity purified by peptide affinity column and used in Western blot analyses.

For Western blots, cells in 6-well plate were washed with PBS and homogenized in 150 μL of lysis buffer containing 1% Triton X-100, 50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 10 mM PMSF (Phenylmethylsulfonyl fluoride), and 2 μg/mL leupeptin. After centrifugation of the homogenate, supernatants were used for analysis.

Construction of Heparanase Promoter−Luciferase Plasmids. The 700 bp human heparanase promoter (−628 to +72, relative to transcription start site) was cloned using the GenomeWalker Kit (Clontech, Palo Alto, CA) and inserted into a luciferase reporter plasmid pGL3Basic (Promega, Medison, WI). The plasmid containing the heparanase promoter was denoted GL3Hep700. In addition, a short fragment of 300 bp of the heparanase promoter upstream of the ATG start codon was obtained by polymerase chain reaction (PCR) and inserted into pGL3Basic, denoted pGL3Hep300.

To mutate the heparanase promoter Sp1 site, two oligonucleotides, 5′-GAGCCGGGCAGGCGGTTCGGGGTTGGATTG-3′ and 5′-CAATCCAACCCC-GAACCGCCTGCCCG GCTC-3′, were used. A mutated Sp1 site of pGL3Hep300, designated pGL3Hep300M, was generated by using PCR-based QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) followed by DNA sequencing to verify the mutated site.

Luciferase Assay. HEK 293 cells were grown to 80% confluency and transfected with pGL3Basic or heparanase promoter-driven luciferase plasmids using Lipofectamine reagent (Invitrogen). After a 20 h incubation, cells were washed and incubated with or without 0.4 mM oleic acid at 37 °C for 4 h. Cell lysates were prepared, and luciferase activity was measured using a luciferase assay system (Promega, Madison, WI).

Electrophoretic Mobility Shift Assay. After incubation of cells with 0.4 mM oleic acid for 6 h, THP1 cells were collected and nuclear extracts were prepared as described in Molecular Cloning (*8*). An oligonucleotide containing the Sp1 site from human heparanase used in this assay is 5′-GGGGCAGGCGGGGCGGGGTTGGG-3′. Double-stranded oligonucleotides were end-labeled with DIG using Roche DIG gel shift kit (Roche, Indianapolis, IN). Two micrograms of nuclear extract protein was used for formation of oligo−protein complexes according to manufacturer's instructions. For a negative control, 125-fold nonlabeled oligonucleotide was added. The reaction mixtures were loaded on NOVEX 6% native DNA retardation gel (Invitrogen), then transferred to nylon membranes, incubated with anti-DIG-AP (Roche) and detected by the CSPD chemiluminescent system.

Immunohistochemical Analysis of Heparanase in Blood Vessels. Aortic tissues from 3-month- or 1-year-old C57BL/6 (wild-type) and apoE-null mice were obtained, fixed in 10% neutral buffered formalin, and embedded in paraffin, and 5 μm sections were prepared for immunohistochemistry. After deparaffinization in xylene and rehydration, sections were treated for antigen retrieval in citrate buffer (0.01 M, pH = 6.0) for 3 min in a microwave oven. Endogenous peroxidase activity was quenched with 1.5% H_2O_2/methanol, and tissues were blocked with 5% normal goat serum to eliminate nonspecific background. Sections were incubated with heparanase antibody (1:140 dilution in 1% BSA/phosphate-buffered saline (PBS)) at 37 °C for 1 h and at 4 °C overnight. After being washed with PBS, sections were treated with biotinylated anti-rabbit IgG, followed by avidin−biotin peroxidase complex (Vector laboratories) at room temperature for 1 h. Colors were developed using aminoethy carbozole as substrate for 1 min. Sections were counterstained with hematoxylin (Zymed). For negative control, primary antibody was replaced by normal rabbit IgG.

Statistical Analysis. Results are presented as mean ± standard deviation. Statistical significance was determined by two-tailed *t* tests. $P < 0.05$ is considered significant.

RESULTS

Ox-LDL and Inflammatory Molecules Induce Endothelial Cell Heparanase. We previously showed that Ox-LDL and its atherogenic component lysolecithin induce the secretion of heparanase activity by bovine endothelial cells (*5*, *6*). To determine whether Ox-LDL induces heparanase in human cells, HMVEC-conditioned media were analyzed for heparanase expression. Western blotting of media using anti-heparanase antibody revealed a single band of ~50 kDa, which was increased 2-fold by Ox-LDL (Figure 1A).

We next determined the effects of other inflammatory cytokines. TNFα increased heparanase secretion by 12-fold, and IL-1β increased heparanase secretion by 8-fold (Figure 1B). In contrast, VEGF and dexamethasone (not shown) decreased heparanase levels in media. Intracellular heparanase was induced by 3-fold and 1.5-fold by TNFα and IL-1β, respectively (Figure 1C), suggesting that the majority

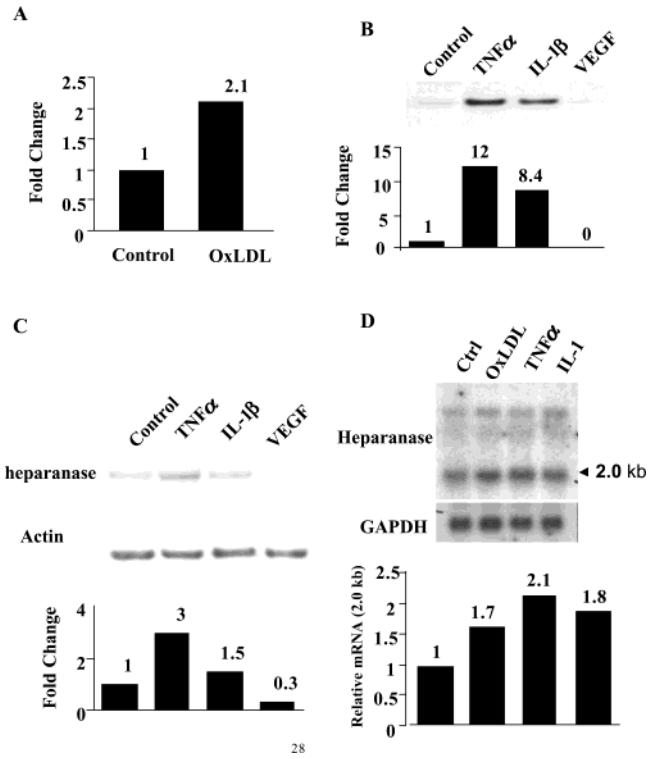

FIGURE 1: Western blot analysis. HMVECs were treated 16−19 h in media containing 1% serum with various stimuli and media, and cell lysates were immunoblotted with anti-heparanase antibody. The fold changes of heparanase secretion, as determined by densitometric analysis, are indicated: (A) induction of heparanase in cell media treated with Ox-LDL (50 μg/mL); (B) regulation of heparanase secretion by TNFα (5 ng/mL), IL-1β (1 ng/mL), and VEGF (200 ng/mL); (C) immunoblot of cytosolic heparanase and actin; (D) northern blot in which 10 μg of total RNAs from untreated and treated cells were used for RNA electrophoresis and membrane was hybridized with human heparanase probe as well as GAPDH probe.

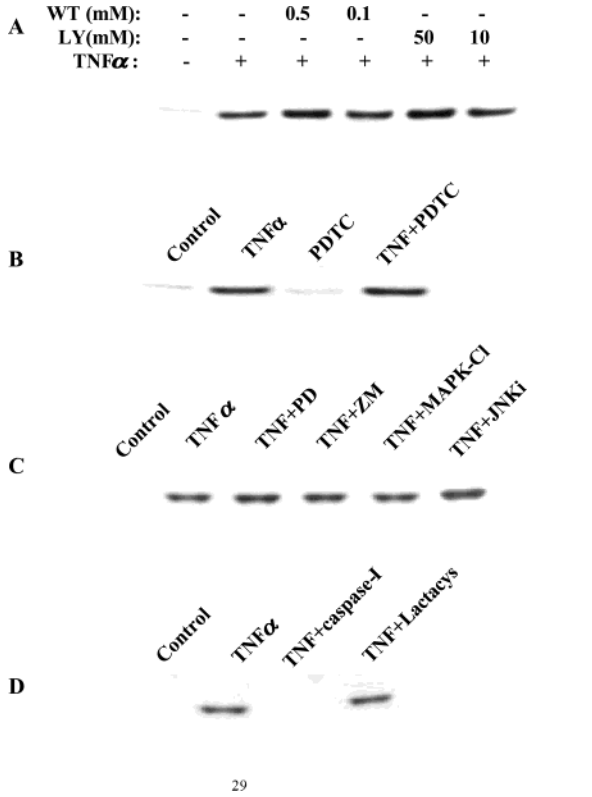

FIGURE 2: HMVECs were treated with TNFα in the presence or absence of inhibitors to various TNFα-induced signaling pathways. Media were then immunoblotted for heparanase protein: (A) PI3-kinase inhibitors wortmannin (WT) and LY294002 (LY) do not block TNFα-induced heparanase secretion; (B) PDTC, an antioxidant inhibitor of NFκB, does not affect TNFα-induced heparanase secretion; (C) inhibitors to the MAPK/JNK signaling pathway do not significantly affect TNFα-induced heparanase secretion; 5 μM MEK inhibitor PD98059 (PD), 200 nM c-Raf inhibitor ZM336372 (ZM), a cocktail inhibitor (CI) that contains four different MAPK cascade inhibitors, or 10 μM JNK inhibitor was used; (D) 10 μM caspase-8 inhibitor abolishes TNFα-induced heparanase secretion; the proteasome inhibitor lactacystin (10 μM) was also used and did not affect TNF-induced heparanase.

of the heparanase induced by these agents was targeted for secretion. TNFα induction of heparanase could be seen with as little as 0.1 ng/mL, and this induction required at least 10 h (not shown). Unlike endothelial cells, neither control nor TNFα-stimulated smooth muscle cells secreted detectable heparanase (not shown).

The changes in heparanase protein were associated with similar changes in mRNA levels, as indicated by Northern blot (Figure 1D). Thus, endothelial cells express an inducible heparanase that is identical to the recently cloned human heparanase.

*Mechanism of Heparanase Induction by TNF*α. We next examined whether nuclear factor-κB (NFκB), a key transcription factor required for expression of many inflammatory genes (*9*), was responsible for heparanase induction. TNFα signaling is a complex process involving several receptor-associated factors and downstream signaling kinases. We tested the roles of two downstream kinase pathways, the PI3-kinase pathway and the MAPK pathway. Neither wortmannin (0.1 μM) nor LY294002 (10 μM), two of the PI3-kinase inhibitors, inhibited TNFα-induced heparanase protein in endothelial cell medium (Figure 2A). At higher concentrations, they further enhanced (30%−50%) the TNFα effect. The NFκB inhibitor, PDTC, failed to inhibit TNFα-induced secretion of heparanase (Figure 2B). Similar results were obtained with SN50, a peptide inhibitor of NFκB (not shown).

Inhibitors of the MAPK/JNK pathway did not significantly affect heparanase induction (Figure 2C). These data suggest that NFκB and PI3-kinase and MAPK/JNK pathways do not directly induce heparanase. To determine the efficacy of these inhibitors, we examined their effect on TNFα-induced expression of cytokine IL-6. SB, MAPK−CI, and LY suppressed TNFα-induced IL-6 by ~80% (data not shown).

*Caspases Are Required for TNF*α*-Induced Secretion of Heparanase.* Caspases are key components of TNFα-induced inflammation and apoptosis. Most cytotoxic effects of TNF are mediated by TNF receptor 1 (TNFR1) through interaction of its death domain with the TNFR-associated death domain protein (TRADD) (*9*). TRADD interacts with Fas-associated death domain protein (FADD) to activate caspase-8, thereby initiating the apoptosis pathway. We tested the effect of caspase-8 inhibitor on TNF-induced heparanase secretion. As shown in Figure 2D, caspase-8 inhibitor completely blocked TNFα-induced heparanase secretion. In contrast, lactacystin, a proteasome inhibitor, had no effect.

Oleic Acid Stimulates Heparanase Expression. To investigate whether FA influence heparanase expression, BAEC were exposed to oleic acid in 1% BSA/DMEM for 6 h. Western blot analysis showed dose-dependent heparanase

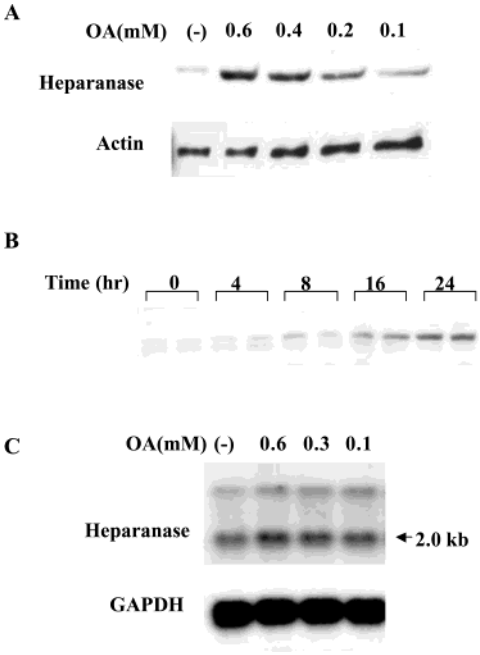

FIGURE 3: Oleic acid dose- and time-dependent stimulated heparanase expression. BAEC were grown to 90% confluency and (A) treated with indicated concentration of oleic acid (0.1−0.6 mM in 1% albumin) for 6 h or (B) treated with 0.4 mM oleic acid cell for indicated times with 1%BSA/DMEM used as control. Cell lysates were used for SDS−PAGE and Western blots performed using anti-human heparanase antibody (1:800) as well as anti-mouse actin antibody (1:3000). In panel C, cells were treated with or without indicated concentrations of oleic acid for 6 h, and RNA was extracted using TRIzol reagent. Northern blot analysis was performed as described in Materials and Methods. Oleic acid increased 2.0 kb heparanase mRNA expression.

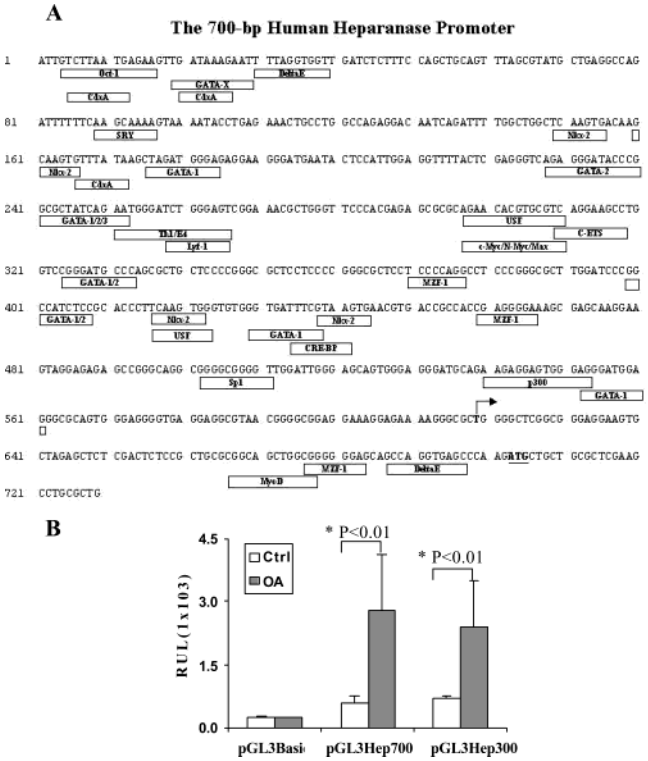

FIGURE 4: Panel A shows the sequence of a 700 bp human heparanase promoter. In panel B, 700 and 300 bp fragments of human heparanase promoter were used to drive a luciferase reporter gene. HEK 293 cells were transfected with control or heparanase promoter-driven luciferase plasmids, then treated with 0.4 mM oleic acid for 4 h. Cell lysates were prepared and luciferase activity was measured as mentioned in Materials and Methods. Oleic acid treatment significantly increased luciferase activity both in 700-bp and in 300-bp heparanase promoter driven constructs ($P < 0.01$).

expression using 0.1−0.6 mM oleic acid (Figure 3A). A time course experiment using 0.4 mM oleic acid showed an increase in heparanase expression beginning at 4 h and increasing over 24 h (Figure 3B). Therefore, heparanase expression is upregulated by oleic acid treatment.

To determine whether oleic acid regulates heparanase expression at the transcriptional level, Northern blot analysis was performed. Heparanase has two mRNA transcripts of 2.0 and 5.0 kb, both reported in human and bovine (*2*, *10*, *11*). Figure 3C shows that cells exposed to 0.4 mM oleic acid had a ∼2-fold increase in 2.0 kb heparanase mRNA content.

Oleic Acid Effects on Heparanase Promoter. To investigate the mechanism responsible for oleic acid stimulation of heparanase expression, a 700 bp fragment upstream of the ATG start codon was cloned by genome walking (Figure 4A). Transcription factor analysis of the promoter showed potential binding sites for several factors, but no NFκB binding elements. We transfected the 700 bp heparanase promoter attached to a luciferase report gene into HEK 293 cells We here chose HEK 293 cells for two reasons: (1) human endothelial cells were difficult to transfect, and (2) HEK 293 cells do not express heparanase (data not shown). Treatment with 0.4 mM oleic acid led to greater luciferase activity than that found in cells only treated with 1% BSA/DMEM (Figure 4B). Because the 300 bp promoter region upstream of the ATG codon had strong basal promoter activity (*15*), we generated the construct only containing this 300 bp fragment. The 300 bp fragment also had greater luciferase activity with oleic acid treatment. Thus, it appeared that an oleic acid regulatory site was present in the first 300 bp region upstream of the translation start site.

Regulatory Mechanism of Oleic Acid on Heparanase Expression. As shown in Figure 4A, human heparanase promoter sequence has an Sp1 binding site 192−201 bp upstream of the ATG initiator. We therefore explored whether oleic acid induced heparanase expression via an Sp1 transcription factor and used a human-derived cell line, THP-1, to do these experiments. First, we confirmed that oleic acid induced heparanase in these cells (not shown). We then performed an electrophoretic mobility shift assay. Figure 5A shows that 0.4 mM oleic acid enhanced Sp1 protein binding activity. This was inhibited by an excess of nonlabeled Sp1-containing oligonucleotide.

Next we examined whether mutating the Sp1 site would reduce the oleic acid induced heparanase promoter activity. Human heparanase Sp1 binding site was mutated from GGGGCGGGGT to GGTTCGGGGT. Luciferase activity assay (Figure 5B) demonstrated that oleic acid induction of the mutant Sp1 heparanase promoter activity of pGL3Hep300M was significantly less than that of non-mutated pGL3Hep300. To further confirm the role of the Sp1 transcription factor in oleic acid induced heparanase expression, BAEC were pretreated with 100 μM mithramycin, then incubated with 0.4 mM oleic acid. Mithramycin partially inhibited heparanase expression induced by oleic acid as demonstrated by Western blots (Figure 5C).

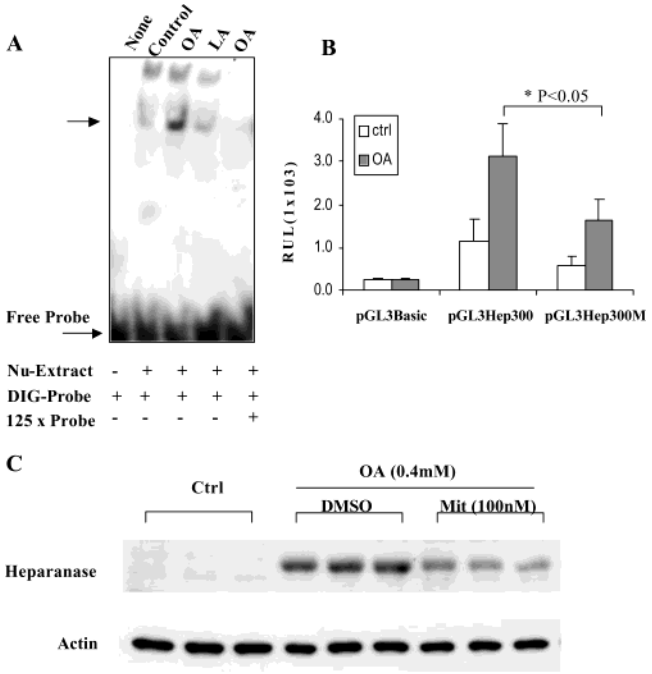

FIGURE 5: In panel A, THP1 cells were incubated without FA (control) or with 0.4 mM oleic acid (OA) or 0.1 mM linoleic acid (LA) for 6 h, and nuclear extracts were prepared. An oligonucleotide containing the Sp1 site from human heparanase was end-labeled with DIG for electrophoretic mobility shift assay as described in Materials and Methods. The final lane on the right shows effects of addition of excess unlabeled Sp1 oligonucleotide. In panel B, luciferase assay was performed as described before; mutant Sp1 (pGL3Hep300M) had significantly less heparanase promoter activity induction by oleic acid than did the nonmutated construct (pGL3Hep300) ($P < 0.05$). In panel C, cells were pretreated with 100 nM mitramycin for 1 h, then incubated with 0.4 mM oleic acid for 6 h. Mithramycin partially inhibited heparanase expression induced by oleic acid.

Heparanase Expression in Blood Vessels. To determine whether heparanase is expressed by endothelial cells in vivo, we performed immunohistochemical analyses of normal and atherosclerotic vessels for heparanase expression (Figure 6). In 3-month-old wild-type mouse aorta, there were no obvious lesions and no immunostaining for heparanase (Figure 6A). In contrast, positive staining was detected in 3-month-old apoE-null mice, prominently in endothelial cells (Figure 6B). Heparanase staining could also be seen in subendothelial matrix, but not in smooth muscle cells. Interestingly, heparanase was only found in atherosclerosis-prone regions of the aortic root, but not in all endothelium. In 1-year-old apoE-null mice, the aorta was thickened with neointima or atheroma formation. At this stage, strong staining for heparanase was found in both endothelial cells and macrophages of the neointima (Figure 6C). However, in advanced lesions of atheroma formation, heparanase staining was limited to the foam cell-rich edges of the atheroma. In 1-year-old control chow-fed, nonatherosclerotic mice, heparanase was not observed (data not shown). Thus, heparanase was localized to both endothelial cells and macrophages in areas of the artery that either have or are prone to the development of atherosclerotic lesions.

DISCUSSION

There are limited data on regulation of heparanase, an enzyme that is likely to be important for a number of physiological processes including response to inflammation, cell migration, and lipid metabolism. Our data show that heparanase is an inflammatory gene product and offer a possible explanation for the decreased HS observed in inflammatory diseases such as atherosclerosis. The heparanase activity previously found to be stimulated by Ox-LDL and lysolecithin in endothelial cell culture medium (*5*, *6*) is probably due to increased secretion of this enzyme, since Ox-LDL increased heparanase protein. Endothelial heparanase is highly induced by inflammatory cytokines, such as TNFα and IL-1β, and down-regulated by VEGF and anti-inflammatory reagents. Heparanase is induced by oleic acid, and this appears to involve an Sp1 site in the promoter region. Finally, we show that endothelial cells in vivo express this enzyme and that it is upregulated in inflammatory regions of blood vessels.

Quiescent endothelial cells produce little heparanase activity (*5*, *16*). Because of the physiological implications of this enzyme in regulation of lipoprotein metabolism and inflammation, we explored whether exposure of endothelial cells to cytokines augmented expression of this enzyme. Both TNFα and IL-1β were potent stimulators of heparanase. Although previous studies by Bartlett et al. showed that TNFα and IL-1 in combination enhanced matrix degrading activity in endothelial cells, the activity was not attributed to heparanase (*37*). Mammalian heparanase was not cloned until 1999 (*1*).

TNFα signaling is a complex process involving several receptor-associated factors and downstream signaling pathways (*17*−*19*). Our data ruled out the participation of several of these pathways in TNFα-induced heparanase secretion. NFκB is a redox-sensitive transcription factor necessary for gene activation in response to inflammatory stimuli such as TNFα and IL-1β. Components of the PI3-kinase and MAPK pathways regulate the activity of NFκB, as well as another inflammation-related factor, AP-1, and enhance gene expression regulated by these transcription factors (*18*−*20*). Data from experiments using specific inhibitors suggest, however, that none of these pathways are critical for heparanase secretion induced by TNFα. Consistent with this, a transcription factor search in a 700 bp functional heparanase promoter revealed no NFκB or AP-1 elements.

Our data clearly showed that caspases play a key role in TNFα-induced heparanase secretion. Caspases are a group of at least 14 enzymes that are thought to be an important part of TNFα-induced inflammatory and apoptotic pathways (*20*). Activation of primarily apoptotic caspases occurs through TNFα receptor-associated death domain protein (TRADD) or Fas-associated death domain protein (FADD), which recruits and activates caspase-8 and initiates a downstream caspase cascade involving caspases-3 and -9 (*9*). This pathway is independent of other kinase-mediated signaling, consistent with our findings that heparanase secretion was only affected by caspase inhibitors but not PI3-kinase and MAPK inhibitors. Analysis of the heparanase amino acid sequence does not reveal potential caspase recognition sites, suggesting that caspases may not play a direct role in heparanase processing but may modulate the activities of other factors that can affect heparanase processing and secretion.

The association between heparanase production and inflammatory cytokines might indicate a pathobiological role

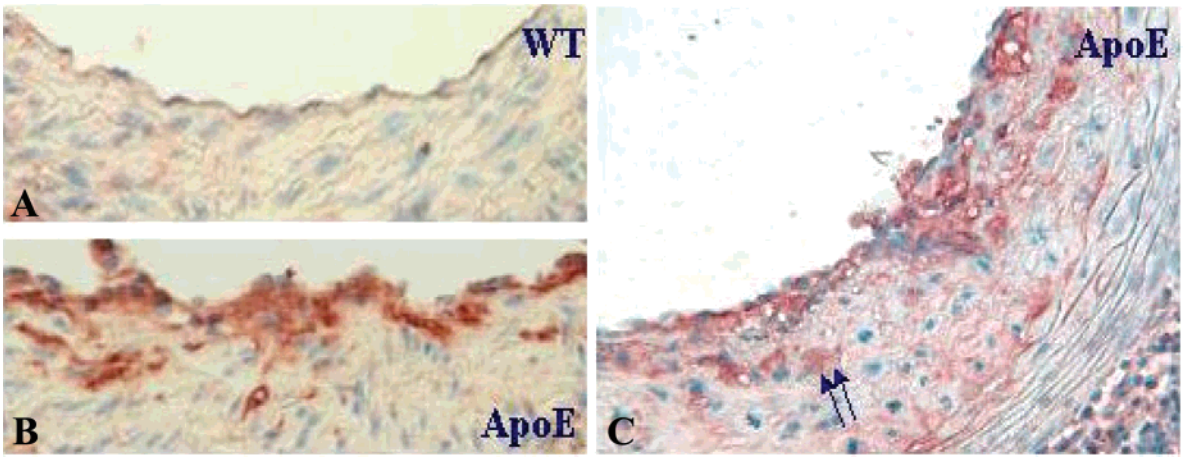

FIGURE 6: Aortic tissue sections from 3-month-old wild-type (A), 3-month-old apoE-null (B), or 1-year-old apoE-null mice (C) were immunostained for heparanase. In advanced lesions, strong staining for heparanase was seen in endothelial cells and also found in macrophages (C, indicated by arrows) of the neointima.

of this enzyme. Subendothelial matrix is a reservoir for several growth factors (*21*) that may be released by heparanase degradation of matrix HSPG. Some of these growth factors may prevent apoptosis. Matrix HSPG plays a critical role in maintaining endothelial integrity, antithrombogenecity and vascular homeostasis (*22*, *23*). Based on previous studies, matrix HSPG appears to be the primary target for heparanase (*5*). Loss of endothelial HS can lead to (1) altered endothelial permeability (*24*), (2) cell proliferation and angiogenesis (*25*), (3) increased monocyte binding to the subendothelial matrix (*16*), and (4) increased smooth muscle cell proliferation (*26*).

Oleic acid also induced heparanase expression; the increase in protein was associated with more heparanase mRNA. Many of the effects of FA on gene expression appear to be mediated by PPAR element activation (*12*−*14*). However, PPARγ agonists, neither 10 μM troglitazone nor 10 μM ciglitazone, did not affect heparanase expression (data not shown). Unlike some FA regulated genes (*12*, *13*), the heparanase promoter has no PPAR elements. Thus, the FA effect was modulated by another transcription site, and we studied the heparanase promoter to determine this site. In agreement with results of others, we found that the first 300 bp proximal to ATG was important for heparanase regulation (*15*); the 300 bp promoter fragment showed only slightly reduced luciferase response to oleic acid treatment compared with the 700 bp fragment. This suggested that FA stimulate heparanase expression via an element within the 300 bp segment upstream of the transcription start site.

Several mechanisms of FA regulation of gene expression have been studied in detail. FA can directly modulate the transcriptional activity of hepatocyte nuclear factor-4α (*27*), S14 promoter elements (*28*), and sterol regulatory element (SRE) (*29*). Moreover, there is a FA response region in stearoyl-CoA desaturase gene 1 promoter (*30*). FA induction of genes via an Sp1 site has also been reported. Human plasminogen activator inhibitor-1 (PAI-1) gene has an Sp1 DNA binding site that is responsive to FA (*31*). In the heparanase promoter sequence, there is an Sp1 binding site 192 bp upstream of the ATG transcriptional initiation site. We showed that this site is likely to be responsible for the oleic acid induction of heparanase by three methods: (1) oleic acid increased the interaction of cell nuclear protein with the heparanase Sp1 sequence; (2) mutating the heparanase promoter Sp1 site reduced the oleic acid induced heparanase promoter activity; (3) the Sp1 binding inhibition drug, mithramycin, suppressed oleic acid stimulated heparanase expression. Some regulatory mechanisms of heparanase gene transcription have been described recently (15,36). Differential transcriptional regulation occurs in different cell types with different physiological and pathophysiological conditions. An SP1 site has been identified by Jiang et al. (*15*) as required for basal expression of heparanase in thyroid tumor cells. However, Egr1, as reported by de Mestre et al. (*36*), is responsible for PMA (Phorbol myristate acetate)-induced heparanase gene transcription in Jurkat T cells. Our results suggest that Sp1 is the oleic acid regulatory target in heparanase promoter.

What are the physiologic implications of FA induction of heparanase? Hydrolysis of HSPG on or in the regions surrounding endothelial cells might affect cell proliferation via growth factors, cell migration, and the actions of some cytokines. The induction of both PAI-1 (*31*) and heparanase should increase the thrombogenesity of the vasculature. One focus of our laboratory has been on the regulation of lipoprotein lipase (LpL), the central enzyme required for conversion of lipoprotein triglyceride into FA (*32*). In our previous work, we discovered that heparanase regulated LpL activity by enhancing LpL release from cultured adipocytes (*5*). Heparanase is preferentially secreted from the basolateral side of polarized cultured endothelial cells. Therefore, in vivo heparanase would be preferentially secreted into the subendothelial space. This allows it to interact with adipocytes and myocytes containing LpL attached to HSPG on their surfaces. We postulate that FA released from initial lipolysis of triglyceride-rich lipoprotein on the adipocyte capillary lumen stimulates the production of the endothelial cell heparanase. This would, in turn, promote the release of more LpL from the adipocyte and myocytes allowing the continuation of the lipolysis reaction.

Inflammatory pathways play a major role in the pathogenesis of atherosclerosis (*33*, *34*). During inflammation, endothelial cells and smooth muscle cells, as well as monocytes/macrophages, secret inflammatory cytokines, such as TNFα and IL-1β, in the vascular wall. Both TNFα and IL-1β antagonists reduce atherosclerotic lesion size in apoE-null mice (*35*). It is possible that heparanase, when induced

by inflammatory cytokines locally in the blood vessel, plays a role in the development of atherosclerotic lesions. Consistent with this, our data show that heparanase expression was absent in wild-type mice but highly induced in apoE-null mice. Heparanase was prominently found in endothelial cells but not smooth muscle cells in early lesions. In advanced lesions, strong staining for heparanase was found in both endothelial cells and macrophages of the neointima. Thus, heparanase might be involved in inflammation or vascular remodeling.

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