Fatty Acids Cause Alterations of Human Arterial Smooth Muscle Cell Proteoglycans That Increase the Affinity for Low-Density Lipoprotein

Mariam Rodríguez-Lee, Gunnel Östergren-Lundén, Boel Wallin, Jonatan Moses, Göran Bondjers, Germán Camejo

Objective—The dyslipidemia of insulin resistance, with high levels of albumin-bound fatty acids, is a strong cardiovascular disease risk. Human arterial smooth muscle cell (hASMC) matrix proteoglycans (PGs) contribute to the retention of apoB lipoproteins in the intima, a possible key step in atherogenesis. We investigated the effects of high NEFA levels on the PGs secreted by hASMCs and whether these effects might alter the PG affinity for low-density lipoprotein.

Methods and Results—hASMC exposed for 72 hours to high concentrations (800 μmol/L) of linoleate (LO) or palmitate upregulated the core protein mRNAs of the major PGs, as measured by quantitative PCR. Insulin (1 mmol/L) and the PPARγ agonist rosiglitazone (10 μmol/L) blocked these effects. In addition, high LO increased the mRNA levels of enzymes required for glycosaminoglycan (GAG) synthesis. Exposure to NEFA increased the chondroitin sulfate:heparan sulfate ratio and the negative charge of the PGs. Because of these changes, the GAGs secreted by LO-treated cells had a higher affinity for human low-density lipoprotein than GAGs from control cells. Insulin and rosiglitazone inhibited this increase in affinity.

Conclusions—The response of hASMC to NEFA could induce extracellular matrix alterations favoring apoB lipoprotein deposition and atherogenesis. (Arterioscler Thromb Vasc Biol. 2006;26:130-135.)

Key Words: proteoglycans ■ smooth muscle cells ■ LDL ■ fatty acids ■ insulin

Insulin resistance (IR) and type 2 diabetes (T2D) are associated with a 2- to 4-fold increase in atherosclerotic coronary artery disease.1,2 Changes in circulating lipoproteins, chronic high levels of albumin-bound nonesterified fatty acids (NEFA, and hyperinsulinemia are important contributors to this association.3-5 Atherogenesis involves a tissue response to deposition of apoB lipoproteins and insulin signaling defects that may affect vascular cells.6 The accelerated intimal thickening of large arteries observed in IR and diabetes includes excessive production of matrix components, such as proteoglycans (PGs) and collagens by smooth muscle cells (SMCs).7 Cell culture experiments suggest that increased endothelial cell permeability and SMC alterations of matrix production could be caused by exposure to excessive amounts of NEFA.8-10

In IR, arterial cells are chronically exposed to increased levels of circulating NEFA. In addition, NEFA could be produced locally by lipolytic degradation of lipoproteins, additionally increasing their concentration in the arterial intima.11,12 Retention of low-density lipoprotein (LDL) in the intima by chondroitin sulfate (CS)-rich PGs appears to be a key step in atherogenesis at sites of intima thickening.13-15 We reported that the expression of genes for extracellular matrix proteins and PGs is increased when human arterial SMCs (hASMCs) are exposed to NEFA. Furthermore, the extracellular matrix produced by the NEFA-treated hASMC had a higher affinity for LDL.9

In this study we describe how linoleate (LO) and palmitate (PA) increased the expression of genes encoding the core proteins of the main secreted PGs from hASMC and of the key enzymes in CS-glycosaminoglycan (GAG) biosynthesis. Exposure to LO also altered the GAG composition. Insulin, dose dependently, attenuated the effects of LO. The results explain why the changes in PG composition and structure increase the affinity of the secreted matrix for LDL and suggest a mechanism through which IR and its dyslipidemia can contribute to atherogenesis.

Methods

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Fatty Acid-Albumin Complex Preparation

Solutions containing sodium LO or PA bound to albumin were prepared from stock solutions of the fatty acid sodium salt and fatty...
acid-free BSA. Agarose gel electrophoresis of final solutions containing 10^5 cpm/μL of ^13C-labeled LO indicated that >99.9% of the radioactivity was bound to albumin.

**Cell Culture**

Primary cultures of hASMCs from inner media of uterine arteries were established by an explantation technique. Cells were grown in medium 199 (M-199) with Earle’s salts, antibiotics, sodium pyruvate, and L-glutamine (M199+) containing 10% (v/v) human serum.

**GAG Biolabeling**

Cells were synchronized in M199+ containing 0.02% human serum, because the amount of GAGs and the length and sulfation degree of their GAG chains may change with proliferation. After 48 hours, the medium was replaced with sulfate-depleted minimum essential medium with 10% (v/v) human serum, antibiotics, sodium pyruvate, and L-glutamine. The next day, radioactive precursors ([35S] Sulfate, D-[6-3H] glucosamine hydrochloride) and BSA, fatty acids, and/or insulin were added. In some experiments, the PPARγ agonist rosiglitazone and the protein kinase C (PKC) inhibitor bisindolylmaleimide I (BIM I) were added together with LO. Media were collected after 72 hours.

**GAG Isolation and Analysis**

GAGs were isolated by precipitation with 95% ethanol and characterized with agarose gel electrophoresis and chondroitinase treatment. Evaluation of the chain length was performed by PAGE. The mRNAs for enzymes involved in CS synthesis were characterized with agarose gel electrophoresis and chondroitinase treatment.

**RT-PCR**

Total RNA was isolated and reversed transcribed. Quantification of mRNA was performed using the ABI PRISM 7700 Sequence Detector (Applied Biosystems). Primer and probe sequences were characterized with agarose gel electrophoresis and chondroitinase treatment. The mRNAs for enzymes involved in CS synthesis were also evaluated.

**Extraction and Analysis of Lipids**

Analyses of SMC lipid classes were performed by high-performance liquid chromatography and evaporative light-scattering mass detection. Conventional Oil Red O staining was performed as described.

**LDL Binding**

LDL (d=1.019 to 1.063 g/mL) was isolated from fresh human plasma by differential ultracentrifugation. Binding of LDL to ^35S/H-labeled GAGs was evaluated by electrophoretic band shift analysis.

**Data Analysis**

All of the densitometric evaluations were done with a Bio-Rad molecular imager system using the Quantity One software. Results are given as means±SE. Differences between 2 groups were identified with a Student t test. Multiple mean values were compared with a 1-way ANOVA and Dunnett’s post hoc test to identify differences versus fatty acid-treated cells. A value of P<0.05 was accepted as statistically significant. The SSPS software was used for statistical analysis.

**Results**

**Effects of High NEFA and Insulin on mRNA of the PG Core Proteins**

Both LO and PA upregulated the mRNA for the core protein of versican, a large CS-containing PG (Figure 1). The mRNA of biglycan, a small leucine-rich PG carrying 2 chondroitin or dermatan sulfate (DS) chains, and that of perlecan, a membrane-bound PG with predominantly heparan sulfate (HS) chains, were also increased compared with controls but to a lesser extent (Figure 1). Decorin expression was increased by LO, but significant differences were not reached. Insulin alone from 1 to 100 nmol/L had no effect on any of the analyzed mRNAs (Table I, available online at http://atvb.ahajournals.org). However, when insulin (10 nmol/L) was added together with the fatty acid, a reduction of the fatty acid-induced upregulation of versican, biglycan, and perlecan mRNAs (50%, 60%, and 74%, respectively) was observed. It should be noted that the basal concentration of insulin in the media, which contained 10% of human serum, was <10 pmol/L. We additionally explored the effect of increasing doses of insulin on the augmented expression of core protein genes for versican and biglycan as induced by LO and PA. The results for versican and biglycan mRNAs indicate that the EC_{50} of insulin for this effect was ≈1 nmol/L (Table II, available online at http://atvb.ahajournals.org).

We also found that the PPARγ agonist rosiglitazone at 10 μmol/L inhibited the LO-induced changes in the mRNA for the core proteins of versican, biglycan, and perlecan. This effect was concentration dependent (Figure 2).
Structural Changes of the PGs Induced by LO

Versican, a PG that can carry ≈12 CS chains with smaller amounts of DS GAGs, is the most abundant PG of those secreted by hASMC in culture. Therefore, the relative content of CS chains secreted by the cells into the media should be augmented, if increased expression of mRNA for the core protein is accompanied by increased secretion of the versican isoforms with GAG attachment domains, V0 or V1. Three isoforms denoted V1, V2, and V3 can be generated by alternative splicing from the larger transcript, V0. These isoforms vary in their relative content of the 2 GAG attachment domains, that is, GAG-α and GAG-β, which are differentially omitted in the V1 and V2 isoforms and are entirely absent in the V3 isoform. Treatment with chondroitinases AC-I and ABC, and subsequent electrophoresis, indicated that CS constitutes 70% to 85% of the secreted GAGs. LO 800 μmol/L increased the (CS+DS):HS ratio 1.5 times the value for control cells. This effect was concentration dependent and observed already with additions of 50 to 100 μmol/L (Figure I, available online at http://atvb.ahajournals.org).

LO Increases Sulfation of Secreted GAGs

Metabolically labeled GAGs from the media were isolated and used for evaluation of binding parameters using increasing amounts of human LDL and electrophoretic band shift analysis. This method allows an estimation of interactions at physiological ionic conditions. The GAGs isolated from LO-treated cells bound more efficiently to LDL than the GAGs from control cells. Insulin (10 nmol/L) and rosiglitazone (10 μmol/L) abrogated this fatty acid-dependent increase (Figure 5). The results suggest that the LO-induced structural changes in GAGs caused a higher affinity for LDL and that these changes and the associated increased affinity were inhibited by insulin and the PPARγ agonist.
zone showed a tendency to decrease the $^{35}$S:$^{3}$H ratio, but the differences did not reach statistical significance.

**Changes in Intracellular Lipid Content**

After incubation with 800 $\mu$mol/L of LO, the triacylglycerol (TAG) content of the cells increased $>$20-fold, from $1.22 \pm 0.20$ to $26.48 \pm 0.70$ $\mu$g/mg of cell protein ($P < 0.05$, $n = 3$). Also, the content of diacylglycerol (DAG) increased significantly from $0.10 \pm 0.01$ to $0.23 \pm 0.02$ $\mu$g/mg of cell protein ($P < 0.05$, $n = 3$). Insulin addition did not alter these values. No increases in other lipid classes were observed (Table III, available online at http://atvb.ahajournals.org). Oil Red O staining revealed lipid droplets in the cytoplasm of fatty acid-treated cells (Figure III, available online at http://atvb.ahajournals.org).

**Discussion**

IR, hyperinsulinemia, and hyperglycemia in T2D have been suggested as agents that could alter PGs of the vascular extracellular matrix. Increased levels of albumin-bound NEFA, a component of the dyslipidemia of IR, have also been suggested to alter the GAGs of the basement membrane of endothelial cells increasing the permeability. However, most of the arterial intima is made of extracellular matrix secreted by SMCs. This is the environment where key initial interactions contributing to atherosclerosis take place. Therefore, it is important to explore whether metabolic alterations associated with IR and T2D can be responsible for the atherogenic changes observed in the human extracellular intima.

The present experiments indicate that linoleic and palmitic acid increased the expression of genes for the core proteins of versican, the small leucine-rich PGs with either 1 (decorin) or 2 (biglycan) chondroitin/DS side chains, and perlecan, the main HS-containing PG of the basement membrane. These NEFA effects were accompanied by an increase of CS-rich PGs secreted into the media. In previous experiments, we found that the extracellular matrix synthesized by cells exposed to fatty acids had a higher affinity for LDL than that of unexposed cells. The present data using band shift analysis, conducted with the isolated secreted GAGs, indicate that this increase is most likely caused by CS-GAG chains contributed by versican and biglycan. Versican is the most abundant PG of the human intima and, when isolated from human arteries or SMC cultures in vitro, shows a high affinity for LDL. Biglycan, on the other hand, has been shown to colocalize with apoB and apoE-containing lipoproteins in human coronary atherosclerotic intima and has been found to bind LDL. Moreover, changes in the composition of arterial GAGs in human diabetes have been detected.

Our data suggest also that the increase in negative charge density of the GAGs caused by the NEFA contributed to the increased LDL binding. This increase in negative charge, indicated by the higher anodic mobility of the CS chains, appears to be a product of an increase of sulfate groups relative to hexosamine units, as indicated by the changes in the $^{35}$S:$^{3}$H ratio. The elevated expression of the genes for CS-4 and CS-6 sulfotransferases, the enzymes that attach negative sulfate groups to the polymerizing GAG chain, observed on LO exposure supports this conclusion. It is important to mention in this context that GAGs isolated from the intima of atherosclerosis-prone human arteries are enriched in highly sulfated CS. These GAGs also show a higher affinity for LDL, a property that, as mentioned, could contribute to increased LDL entrapment in lesion-prone arteries.

Intramyocellular TAG levels correlate tightly with the severity of IR. In addition to inducing TAG biosynthesis, NEFA also stimulates the synthesis of other less abundant metabolites, such as DAG and ceramides. Both derivatives of fatty acyl CoA have been implicated as primary mediators of the antagonistic effects of NEFA in skeletal muscle. DAG is hypothesized to activate a signaling cascade leading to the inhibition of IRS-1 whereas ceramides have been shown to block activation of Akt/protein kinase B. As a result of increased intracellular DAG, PKC is chronically
activated in diabetes and nondiabetic IR. Also, high-glucose concentrations or diabetes cause increased activity of membrane-associated PKC and are associated with increased intracellular DAG concentrations in several tissues and in cultured aortic endothelial cells and vascular SMCs.\textsuperscript{45–47} Furthermore, in skeletal muscle of PKC\textbeta knockout mice, PKC\textbeta activation during hyperlipidemia was necessary for the inhibition of skeletal muscle IRS-1 tyrosine phosphorylation and insulin-stimulated glucose uptake.\textsuperscript{48} We found that incubation with LO increased the cellular content of TAG and DAG compared with control cells. In addition, BIM I, a nonspecific PKC inhibitor, blocked the effects of linoleic acid on the (CS + DS):HS ratio of secreted GAGs, suggesting that activation of PKC by fatty acids could be the cause of impairment of insulin signaling involved in PG and GAG biosynthesis, possibly by increasing the intracellular DAG content.\textsuperscript{49} Thus, it is possible that in our experiments NEFA weakens insulin signaling by activation of PKC.

In addition, we found that the PPAR\gamma agonist rosiglitazone blocked the effects of LO on PGs, GAGs, and LDL binding. However, we did not find a consensus peroxisome proliferating response element in the upstream sequence spanning 6000 bases before the starting codon in any of the genes screened. Because PPAR\gamma is expressed in SMCs, we speculate that it may oppose the actions of NEFA by accelerating their conversion into stored TAGs. This could limit the concentration of DAG or other bioactive lipids that could modulate the insulin-signaling cascade.\textsuperscript{50} It may also limit the actions of fatty acyl-CoA on glucose use and lower the contribution of the hexosamine pathway. The end products of this pathway, UDP-Glc-NAc and UDP-Gal-Nac, are the building blocks for the synthesis of the GAG chains of PGs. This action could explain why rosiglitazone inhibited the effects of NEFA in a dose-dependent manner.

Under our culture conditions, insulin on its own added on top of the basal level of <10 pmol/L had no effect on any of the PG or GAG properties. On the other hand, the changes caused by the fatty acids on genes encoding the PG core proteins, the substantial changes in GAG structure, and increased affinity for LDL were blunted with insulin levels >1 nmol/L. These results suggest that levels of insulin in the picomole range are sufficient to maintain a basal rate of PG synthesis and composition. However, under overexposure to NEFA, 1 to 5 nmol/L of insulin was required to restore the basal biosynthesis and structure of secreted PGs. In hASMCs, it has been reported previously that the insulin effects on 2-deoxy-D-glucose transport have an approximate \( K_0 \) of 25 nmol/L.\textsuperscript{51} Arterial SMCs are responsive to both insulin and IGF-I, and their effects are additive at near physiological concentrations.\textsuperscript{52,53} Both ligands can cross-react with the receptors of the other but with 100- to 1000-fold less potency than to their own receptors.\textsuperscript{54} Insulin receptors are few in SMCs, whereas the IGF-I receptor is highly expressed in SMCs in intact arteries and in cultured SMCs.\textsuperscript{54} Insulin and IGF-I receptors also show great similarities in their signaling pathways. Our results, indicating that 1 to 5 nmol/L of insulin is required to abrogate the fatty acid effects on secreted PGs, may indicate that the hormone is acting via both receptors pathways. It is important to stress that this is a direct possible beneficial effect of insulin on hASMC but that another important one present in vivo is the capacity of the hormone to reduce circulating NEFA levels.\textsuperscript{3,55}

In summary, the current study indicates that exposure of hASMC to high levels of fatty acids can cause structural alterations of secreted PGs that explain the increased binding of LDL. Such effects were blunted by insulin and PPAR\gamma activation. If similar effects are present in vivo in conditions of IR, overexposure of the arterial smooth muscle to fatty acids could induce extracellular matrix changes favoring apoB lipoprotein deposition and atherogenesis.

Acknowledgments

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Table I.

Insulin by itself had no effect on PG core protein expression. Expression of PG core proteins were determined after exposure of hASMCs to 300 µmol/l BSA (control) and 1, 10 or 10 nmol/l insulin for 72h. Total RNA was isolated, and mRNAs for specific proteins were quantified. Data presented is corrected for β-actin expression. Values are means ± SE (n=4). One-way ANOVA was used to compare means of independent groups. No significant differences were found.

<table>
<thead>
<tr>
<th></th>
<th>versican</th>
<th>biglycan</th>
<th>perlecan</th>
<th>decorin</th>
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</thead>
<tbody>
<tr>
<td><strong>BSA 300µM</strong></td>
<td>0.60 ± 0.12</td>
<td>0.81 ± 0.10</td>
<td>0.52 ± 0.04</td>
<td>0.83 ± 0.08</td>
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<tr>
<td><strong>Ins 1nM</strong></td>
<td>0.51 ± 0.11</td>
<td>0.74 ± 0.16</td>
<td>0.52 ± 0.17</td>
<td>0.85 ± 0.23</td>
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<tr>
<td><strong>Ins 10nM</strong></td>
<td>0.49 ± 0.13</td>
<td>0.69 ± 0.07</td>
<td>0.49 ± 0.11</td>
<td>0.86 ± 0.04</td>
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<tr>
<td><strong>Ins 100nM</strong></td>
<td>0.62 ± 0.14</td>
<td>0.72 ± 0.02</td>
<td>0.52 ± 0.03</td>
<td>0.83 ± 0.18</td>
</tr>
</tbody>
</table>
Table II.

Effect of insulin on the fatty acid-induced expression of versican and biglycan core proteins. Data are normalized to β-actin expression and presented as means ± SE, n=3 for linoleate and n=4 for palmitate. Significance was tested versus the fatty acid addition, *P<0.05.

<table>
<thead>
<tr>
<th></th>
<th>versican linoleate</th>
<th>versican palmitate</th>
<th>biglycan linoleate</th>
<th>biglycan palmitate</th>
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</thead>
<tbody>
<tr>
<td><strong>BSA 300µM</strong></td>
<td>1.55 ± 0.03 *</td>
<td>0.97 ± 0.03 *</td>
<td>1.89 ± 0.06 *</td>
<td>0.76 ± 0.04 *</td>
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<tr>
<td><strong>FA 800µM</strong></td>
<td>2.95 ± 0.11</td>
<td>1.95 ± 0.08</td>
<td>3.48 ± 0.32</td>
<td>1.26 ± 0.05</td>
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<tr>
<td>FA+Ins 0.1nM</td>
<td>-</td>
<td>1.51 ± 0.08 *</td>
<td>-</td>
<td>1.02 ± 0.04 *</td>
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<tr>
<td>FA+Ins 1nM</td>
<td>1.71 ± 0.16 *</td>
<td>1.20 ± 0.06 *</td>
<td>2.60 ± 0.13</td>
<td>0.98 ± 0.01 *</td>
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<tr>
<td>FA+Ins 10nM</td>
<td>1.09 ± 0.11 *</td>
<td>1.13 ± 0.02 *</td>
<td>2.23 ± 0.41 *</td>
<td>0.85 ± 0.04</td>
</tr>
<tr>
<td>FA+Ins 100nM</td>
<td>0.97 ± 0.05 *</td>
<td>-</td>
<td>2.22 ± 0.06 *</td>
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</tr>
</tbody>
</table>
Figure I.

Linoleate dose-dependently increased the (CS+DS)/HS ratio of GAG chains from secreted PGs. HASMCs were exposed to culture medium containing increasing concentrations of linoleate: 50, 100, 200, 400 or 800 µmol/l. (CS+DS)/HS ratios were determined after releasing the media $^{35}$S/$^3$H-GAGs by proteolysis and electrophoretic separation. Autoradiographs of the gels were evaluated by densitometric analysis. Values are means ± SE (n=3).

*P<0.05 versus control cells (BSA 300 µmol/l), as analyzed with ANOVA and Dunnett’s test.
Figure II.

Treatment with the PKC inhibitor, BIM I, opposed linoleate-induced changes in (CS+DS)/HS ratio of secreted GAG chains. Only the results obtained with 300 µmol/l LO are shown. Data are presented as % of control (BSA 300 µmol/l). Values are means ± SE (n=3). *P<0.05 versus linoleate-treated cells, as analyzed with ANOVA and Dunnett’s test.
Table III.

Analysis of SMC lipid classes by high performance liquid chromatography and evaporative light scattering mass detection. After incubation with 800 µmol/l LO the triacylglycerol (TAG) and diacylglycerol (DAG) content of the cells increased significantly compared to control cells (300 µmol/l BSA). However no increases in cholesterol esters (CE), free cholesterol (Chol) or phospholipids were detected. Differences between the two groups were identified with a Student’s t test. A value of $p<0.05$ was accepted as statistically significant. Phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl choline (PC) and lysolecithin (LL).

<table>
<thead>
<tr>
<th></th>
<th>BSA 300µM</th>
<th>LO 800µM</th>
<th>P value</th>
</tr>
</thead>
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<td>CE</td>
<td>8.85 ± 1.70</td>
<td>7.49 ± 0.70</td>
<td>0.5007</td>
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<tr>
<td>Chol</td>
<td>3.78 ± 0.30</td>
<td>2.51 ± 0.04</td>
<td>0.0141</td>
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<tr>
<td>PE</td>
<td>7.81 ± 0.40</td>
<td>5.63 ± 0.40</td>
<td>0.0175</td>
</tr>
<tr>
<td>PS</td>
<td>3.52 ± 1.10</td>
<td>2.41 ± 0.30</td>
<td>0.4330</td>
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<tr>
<td>PC</td>
<td>9.57 ± 2.00</td>
<td>7.60 ± 0.30</td>
<td>0.3740</td>
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<tr>
<td>LL</td>
<td>2.91 ± 0.50</td>
<td>3.50 ± 1.00</td>
<td>0.6245</td>
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</tbody>
</table>
Figure III.

**Oil Red O staining.** HASMCs were exposed to control medium, 300 μmol/l BSA (a), or 800 μmol/l LO (b) during 72 h. Lipid droplets were detected in the cytoplasm of fatty acid-treated cells but no morphological changes consistent with more pronounced foam cell formation were observed. A representative picture from 3 individual replicates is shown (Original magnification x 100).
METHODS (full-length version)

Materials. Sodium linoleate and palmitate, fatty acid-free BSA, recombinant human insulin, HEPES, papain and bisindolylmaleimide I (BIM I) were from Sigma-Aldrich. NuSieve 3:1 agarose was acquired from Cambrex Bioscience. [35S] Sulfate and D-[6-3H] glucosamine hydrochloride were obtained from Amersham Bioscience. Centricon YM-3 units were from Millipore. Chondroitinases AC-I and ABC, heparitinase III and chondroitin sulfate A (CS4) were from Seikagaku. Rosiglitazone was a gift from AstraZeneca (Mölndal, Sweden). Primers for reverse transcriptase (RT)-polymerase chain reaction (PCR) and sulfate-depleted Eagle’s minimum essential medium (MEM) were from Invitrogen Life Technologies. Reagents for RT and real-time PCR, fluorescently labeled probes, TaqMan Endogenous Control and Assays-on-Demand were from Applied Biosystems. Medium 199 with Earle’s Salts (M199), trypsin, antibiotics, L-glutamine, sodium pyruvate, Dulbecco’s phosphate-buffered salt solution (DPBS), fetal bovine serum (FBS) and human blood serum were from PAA Laboratories. All other chemicals were of analytical grade and obtained from VWR International.

Fatty Acid-Albumin Complex Preparation. In order to avoid the addition of organic solvents to the cell culture medium, solutions containing sodium linoleate or palmitate bound to albumin were prepared from stock solutions of the fatty acid sodium salt (16 mmol/l) and fatty acid-free BSA (6
mmol/l). In brief, 75 mg of LO-Na salt were dissolved in 3 ml sterile \( H_2O \) at 37°C and 1.94 ml of this solution was added to 8.06 ml cell culture media (see Cell Culture), containing 6 mmol/l BSA. Albumin-bound palmitate was prepared in the same fashion. These stock solutions were used after filtration through 0.22-\( \mu \)m filters and the final fatty acid concentration was measured (NEFA-C kit from Wako). Agarose gel electrophoresis of the final solutions containing \( 10^5 \) cpm/\( \mu l \) of \( ^{14}C \)-labeled LO indicated that >99.9% of the radioactivity was bound to albumin. To evaluate the purity of the fatty acid-free BSA, we performed PAGE 10% under reducing conditions followed by Coomasie Blue staining and densitometric evaluation. No contaminants were detected. The BSA monomer, dimer and trimer band corresponded to more than 99.5% of total protein staining.

**Cell Culture.** Primary cultures of human arterial smooth muscle cells (hASMCs) from inner media of uterine arteries were established by an explantation technique.\(^1\) Cells used were in the third to tenth passage. Cells were grown under normal cell culture conditions (37°C, 21% \( O_2 \), 5% \( CO_2 \), 74% \( N_2 \)) in M199 supplemented with 10% (v/v) human serum, 100 U/ml penicillin, 100 \( \mu g/ml \) streptomycin, 2 mmol/l sodium pyruvate and 2 mmol/l L-glutamine. M199 contained 5.6 mmol/l glucose that corresponds to a normal human fasting glucose level. Insulin in media was measured by a radioimmunoassay (Linco Research). Incubation conditions did not affect cell viability or proliferation.

**GAG Biolabeling.** Two days after seeding, cells were synchronized in M199 containing 0.02% human serum, antibiotics, sodium pyruvate and L-glutamine since the amount of PGs, the length of their GAG chains and their degree of sulfation may change with proliferation.\(^2\) After 48 h, the medium was replaced with sulfate-depleted MEM supplemented with 10% (v/v) human serum, antibiotics, sodium pyruvate and L-glutamine. Next day, fresh sulfate-depleted
MEM 10% human serum was given to the cells, with the addition of 300 µmol/l BSA, fatty acids and/or insulin. Insulin concentrations of 0.1, 1, 10 and 100 nmol/l and LO concentrations of 50, 100, 200, 400 and 800 µmol/l were used. When added, the PPARγ agonist rosiglitazone and the protein kinase C (PKC) inhibitor BIM I were supplemented together with LO. At the same time, cells were exposed to 50 µCi/ml [35S] Sulfate and 10 µCi/ml D-[6-3H] glucosamine hydrochloride. After 72 h of incubation the cultures retained their sub-confluent state and media were collected including complete protease inhibitor cocktail (Roche Diagnostics).

**GAG Isolation and Analysis.** Medium was filtrated through a 0.22-µm filter and the total cell protein was determined with the Modified Lowry Protein Assay Kit (Pierce Biotechnology). Cold carrier (100 µg CS4) was added and samples were filtered through a Centricon YM-3 unit. GAG chains were released from the PG core protein by overnight digestion with papain at 60°C. GAGs were then isolated by precipitation with 95% ethanol and characterized with agarose gel electrophoresis and chondroitinase treatment as described. Evaluation of the length of isolated GAG chains was made by polyacrylamide gel electrophoresis (PAGE). Aliquots containing equal amounts of incorporated D-[6-3H] glucosamine were used for evaluation of GAG composition and chain length.

**RT-PCR.** After incubations, cells were disrupted in lysis buffer and total RNA was isolated (RNEasy Minikit, Qiagen). The RT step was performed at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Samples not treated with retrotranscriptase were included to exclude genomic DNA contamination. The corresponding cDNA(s) to individual mRNA(s) for PG core proteins were evaluated using the fluorogenic 5’ nuclease assay (TaqMan) in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) for real-time PCR as
described. Primer pairs (sense / antisense) and probes were designed using the software Primer Express 1.5 (Applied Biosystems) to target four PG core protein mRNAs. Each probe was labeled at the 5’ end with a fluorescent reporter dye (6-carboxy-fluorescein, FAM) and contained a quencher dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3’ end. Sequences were as follows:

- biglycan GTCTCTGCTGGCCCTGAGC / TCATGAATGGCCCATCGTC and probe CCCTGCCCTTTGAGCAGAGAGGC;
- decorin AAGGCCACTATCATCCTTCT / AAAGTCAAATAAGCCTCTCTGTGAAA and probe TTGCACAAGTTTCTCGGCCCGC;
- perlecan TGGCTGACAGCATCTCAGGA / CGATGGAGCGAGTGAAATTCA and probe ACTTCCAGATGTTTATTTCCGAGCCTG;
- and versican ACGATGTGTATTGTTATGTGGATCATCT / AGCCTCCTCGAAGGTGAATTTA and probe TGATGTGTTCCACCTCACTGTCCCCA.

Primers for human versican were placed in a region common to all splice variants. Amplification product sizes were estimated by standard PCR and electrophoresis. We performed primers, probe and template optimization steps to determine the conditions providing the highest efficiency for the real time PCR reactions. The mRNAs for 3 enzymes involved in CS synthesis were also evaluated: CS-synthase, CS-4 and CS-6 sulfotransferases (Assays-on-demand: Hs00208704_m1, Hs00218229_m1 and Hs00427946_m1, respectively).

Target and endogenous control amplifications were performed in separate tubes using the standard curve method for relative quantitation. A pre-developed primer/probe mix for human beta-actin (4310881E) was used as endogenous control to normalize target mRNA values for differences in the amount of total RNA added to each reaction. All samples were analyzed in triplicate real time PCR reactions.
Extraction and Analysis of Lipids. Analyses of smooth muscle cells lipid classes was performed by high performance liquid chromatography and evaporative light scattering mass detection after extraction of the lipids in the cell layer with chloroform:methanol (2:1). Conventional Oil Red O staining was performed as described. In brief, cells were fixed in DPBS 4% formaldehyde for 10 minutes, stained with 0.5% Oil Red O for 15 minutes, differentiated in isopropanol 60% and counterstained with Mayer hematoxylin for 5 minutes.

LDL Binding. LDL (d=1.019-1.063 g/ml) was isolated from fresh human plasma of healthy, fasting males by differential ultracentrifugation. Binding of LDL to \( \text{^{35}S/^{3}H} \)-labeled GAGs was evaluated by electrophoretic band shift of the complexes at physiological pH and ionic strength. Equal amounts of GAGs, as determined by D-[6-\(^{3}H\)] glucosamine content, were incubated with increasing amounts of LDL for 1h at room temperature and electrophoresed. Evaluation of the gels was done by autoradiography. Data were fitted to one-site hyperbola binding curves and apparent dissociation constants (app\(K_D\)) and maximum binding (B\(_{MAX}\)) were obtained.

Data Analysis. All densitometric evaluations were done with a Bio-Rad molecular imager system using the Quantity One software (Bio-Rad). Binding curves were fitted with the GraphPad Prism software. Results are given as means ± SE. Differences between two groups were identified with a Student’s \(t\) test. Multiple mean values were compared with a one-way analysis of variance (ANOVA). When the overall \(F\) test was significant, differences between groups were further explored with posthoc multiple comparisons (Dunnett’s test) taking fatty acid-treated cells as the reference group. A value of \(p<0.05\) was accepted as statistically significant. The SSPS software was used for statistical analysis.
References:


