Fatty Acids Modulate the Composition of Extracellular Matrix in Cultured Human Arterial Smooth Muscle Cells by Altering the Expression of Genes for Proteoglycan Core Proteins

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In diabetes-associated microangiopathies and atherosclerosis, there are alterations of the extracellular matrix (ECM) in the intima of small and large arteries. High levels of circulating nonesterified fatty acids (NEFAs) are present in insulin resistance and type 2 diabetes. High concentrations of NEFAs might alter the basement membrane composition of endothelial cells. In arteries, smooth muscle cells (SMCs) are the major producers of proteoglycans and glycoproteins in the intima, and this is the site of lipoprotein deposition and modification, key events in atherogenesis. We found that exposure of human arterial SMCs to 100–300 µmol/l albumin-bound linoleic acid lowered their proliferation rate and altered cell morphology. SMCs expressed 2–10 times more mRNA for the core proteins of the proteoglycans versican, decorin, and syndecan 4 compared with control cells. There was no change in expression of fibronectin and perlecan. The decorin glycosaminoglycan chains increased in size after exposure to linoleic acid. The ECM produced by cells grown in the presence of linoleic acid bound ^{125}I-labeled LDL more tightly than that of control cells. Darglitazone, a peroxisome proliferator-activated receptor (PPAR)-γ ligand, neutralized the NEFA-mediated induction of the decorin gene. This suggests that some of the NEFA effects are mediated by PPAR-γ. These actions of NEFAs, if present in vivo, could contribute to changes of the matrix of the arterial intima associated with micro- and macroangiopathies. *Diabetes* 48:616–622, 1999

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apo, apolipoprotein; BSA, bovine serum albumin; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; FCS, fetal calf serum; FFA, free fatty acid; G-3-PDH, glyceraldehyde-3-phosphate dehydrogenase; Ig, immunoglobulin; K_D, dissociation constant; NEFA, nonesterified fatty acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; SMC, smooth muscle cell; UDP-GalNAc, uridine-diphosphate-*N*-acetyl-galactosamine; UDP-GlcNAc, uridine-diphosphate-*N*-acetyl-glucosamine.

Diabetes is associated with changes in the extracellular matrix (ECM). These changes may lead to microvascular complications, such as nephropathy, and the degenerative diseases secondary to diabetes, such as diabetic retinopathy (1). Atherosclerosis is a macrovascular process accelerated by diabetes (2). Changes in the ECM of large vessels in diabetic subjects could also make them more susceptible to this disease (3). Frequently, excess circulating nonesterified fatty acids (NEFAs) precede the development of diabetes (4). Recently, Roden et al. (5) demonstrated that high NEFAs in vivo, in humans, reduce glucose uptake and glycogen synthesis. They suggested that the mechanisms involved inhibition of glucose transport and phosphorylation. Additionally, Hawkins et al. (6) proposed that the reduction in glycolysis rate induced by NEFA infusion and the resulting accumulation of fructose-6-phosphate intracellularly may cause a shift toward the hexosamine biosynthetic pathway in muscle. The end products of this biosynthetic pathway, uridine-diphosphate-*N*-acetyl-glucosamine (UDP-GlcNAc) and uridine-diphosphate-*N*-acetyl-galactosamine (UDP-GalNAc), are substrates for building glycoproteins, proteoglycans, and glycolipids. This seems a plausible mechanism by which chronic exposure to high levels of free fatty acids (FFAs) could affect the synthesis of ECM proteins and ECM proteoglycans. The ECM produced by smooth muscle cells (SMCs) in the intima is the site for deposition and modification of apolipoprotein (apo)B-containing lipoproteins, processes that are critical for atherogenesis (7,8). Intima ECM proteoglycans bind LDLs through specific sequences in apoB-100 (8–11). This interaction seems to lead to the retention of LDL in the arterial wall.

The ECM of the arterial intima controls growth and maintains the tissue architecture (12). Hennig and colleagues (13,14) found that exposing endothelial cells to NEFAs caused alterations in the composition of the synthesized ECM proteoglycans and increased the permeability of the cell monolayer. In small and large arteries, most of the intima ECM is synthesized by arterial SMCs (12). Therefore, if chronic high levels of NEFAs also affect the ECM structure of these cells, such action may contribute to microangiopathies and to atherosclerosis. In the present study, we evaluated the effects of NEFAs on the expression of genes coding for ECM proteoglycans and proteins in human arterial SMCs. We also evaluated the effects of NEFAs on the gene products. Additionally,

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we explored the effect of these alterations on LDL binding properties of the ECM produced by the cells.

RESEARCH DESIGN AND METHODS

Oleic acid, linoleic acid, bovine serum albumin (BSA) grade V, Chondroitinase ABC, HEPES, and *p*-nitrophenylphosphate were from Sigma (St. Louis, MO). Heparitinase was from ICN (Aurora, OH). NuSieve 3:1 agarose was from FMC BioProducts (Rockland, ME). Disposable PD 10 columns (Sephadex G-25) and protein A-Sepharose were from Pharmacia (Uppsala, Sweden). Trypsin and cell culture media were from Biowhittaker (Verviers, Belgium). Fetal bovine serum (FBS) was from Biochrom KG (Berlin, Germany). Human blood serum was from young healthy donors. SDS-PAGE gels (4–12%) were purchased from Novex (San Diego, CA). Anti-human decorin polyclonal antibodies were from Chemicon (Temecula, CA). Anti-human fibronectin monoclonal antibodies (ascites fluid) that do not cross-react with bovine fibronectin were purchased from Life Technologies (Gaithersburg, MD). Normal goat serum, rabbit immunoglobulin (Ig) fraction, mouse IgG1, and alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse antibodies were from Dako (Glostrup, Denmark). Monoclonal antibodies specific for syndecan 1 were purchased from Serotec (Oxford, U.K.). [^{32}P]dCtp, [^{35}S]sulfate, [^{35}S]methionine, and [^{125}I] were purchased from Amersham (Buckinghamshire, U.K.). Scintillation fluid was from Beckman Instruments (Fullerton, CA). All other chemicals were from Merck (Darmstadt, Germany). Darglitazone was a gift from Astra Hässle (Mölndal, Sweden).

Lipoproteins. LDL (d = 1.019–1.063 g/ml) was isolated from fresh human plasma by using differential ultracentrifugation and was stored and labeled with [^{125}I]iodide as described elsewhere (15).

Cell culture. Untransformed mycoplasma-free human arterial SMCs were cultured as previously described (16). Experiments were carried out with cells in the third to tenth passage. Cells were grown in Eagle's minimum essential medium (EMEM) with 10% (vol/vol) FBS with the addition of glutamine, sodium pyruvate, and antibiotics. To have a system in which all cells were proliferating and metabolically active, all experiments were performed in sparse cultures in the presence of 10% FBS. For incubations with NEFAs, media were prepared according to Ramasamy et al. (13). In brief, to EMEM with 10% FBS, 100 µmol/l BSA was added. FFAs were dissolved in a small volume of hexane, precipitated by dropwise addition of 6 mol/l NaOH, evaporated under nitrogen to a thin film, and dissolved in 1 ml of warm water. NEFA suspended in water was added dropwise to the media with stirring. The pH was adjusted to 7.4 with NaOH, and the media were filtered through a 0.22-µm filter. NEFA content in the media was determined with a colorimetric kit from Wako (Neuss, Germany). In the experiments to be reported, we used linoleic acid or oleic acid, because these are two of the most abundant NEFAs present in human plasma (17). With 10% fetal calf serum (FCS) and with 100 µmol/l of BSA added, essentially all NEFA is bound to albumin (18). EMEM from Biowhittaker contains 5.6 mmol/l glucose that corresponds to a normal fasting glucose level. With dilution caused by the additions, the final glucose concentration was 5.4 mmol/l.

NEFA content in the media was checked after incubation with cells. Because the changes observed were within 5%, the indicated NEFA concentrations are the added NEFA and are not corrected for product loss during preparation or concentration caused by evaporation. To measure possible oxidation of NEFA, conjugated diene content was determined by measuring UV absorbance at 234 nm (19). No evidence of NEFA oxidation was observed in the incubation conditions used, probably because of the presence of albumin and phenol red in the media.

Cell proliferation. To evaluate growth rate, we used a colorimetric cell proliferation enzyme-linked immunosorbent assay (ELISA) kit measuring 5-bromo-2′-deoxyuridine, BrdU, incorporation during DNA synthesis (Boehringer Mannheim, Mannheim, Germany).

Glucose uptake. Uptake of 2-deoxy-D-[^{3}H]glucose was measured essentially as described previously, and radioactivity was measured in a scintillation counter (Wallac YO, Åbo, Finland) (20).

Semiquantitative evaluation of mRNA with the polymerase chain reaction. After incubations with NEFAs, cells were detached by a brief (5-min) incubation with Trypsin, collected in cold phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+}, and immediately put on ice. Cells were pelleted, and a kit was used to prepare total RNA (InVitroGen, Leek, The Netherlands). A polymerase chain reaction (PCR) method for estimating mRNA concentrations based on measuring the concentration of product accumulating in consecutive cycles (21) was used. In all samples, mRNA for glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) was determined and used as a control. The following primers were used: G-3-PDH (22) sense CCCTTCATTGACCTCAACTACATGG (position 166–190) and antisense CATGGTGGTGAAGACGCCAG (position 356–375), defining a fragment of 210 nucleotides; fibronectin (23) sense GCCTGGTACA-GAAATATGTAGTG (position 3908–3929) and antisense ATCCCAGCT-GATCAGTAGGCTGGTG (position 4303–4327), defining a fragment of 420 nucleotides; collagen IV (24) sense TACTCTTTGCTCTACGTGCAA (position 67–87) and antisense CATCACAAAAGAGTAGCCGAT (position 391–411), defining a fragment of 345 nucleotides; versican sense ATCCTTCCCCGGGCCACCA (position 148–166) and antisense ACAGCCTCGGGATGTGTGG (position 577–595), defining a fragment of 448 nucleotides; syndecan 4 sense AGTC GATCCGAGAGACTGAGGT (position 82–103) and antisense CCAGGACCTC CGTTCTCTCAAA (position 447–468), defining a fragment of 387 nucleotides; decorin sense CATGAAGGCCACTATCATCC (position 246–265) and antisense CGTTCCAACTTCACCAAAGG (position 1461–1480), defining a fragment of 399 nucleotides; and perlecan sense CTTCTATTGGTCCCGTGAGG (position 5201–5220) and antisense GTCCTCTCTGGGCTCACTTG (position 5955–5974), defining a fragment of 775 nucleotides.

Immunoprecipitation. Decorin and fibronectin were immunoprecipitated, and the precipitates were processed as described by Whitelock et al. (25). To correct for differences in cell number, cells were lysed by addition of PBS with 0.1% Triton X-100, and scintillation fluid was added to aliquots of the lysed cells, and the mixtures were counted. The incorporated radioactivity was used as an estimate of the number of cells, and the volumes of media used in the immunoprecipitation experiments were corrected accordingly.

Detection of cell-surface syndecan 1 and fibronectin. The amount of syndecan 1 at the cell surface was determined on cells in 96-well plates by means of an ELISA (26). In brief, cells were washed once in Tris-buffered saline (10 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.4), fixed for 30 min in 2% HEPES-buffered paraformaldehyde, blocked with 5% goat serum, and incubated for 1 h with 0.15 µg/ml monoclonal antibody specific for syndecan 1 or fibronectin or mouse IgG1. Rates of change in absorbance were determined in a microplate reader (Molecular Devices, Sunnyvale, CA). We did not use antibodies specific for syndecan 4, but the expression of syndecans 1 and 4 have been shown to be correlated (26).

LDL binding to ECM. ECM was prepared as described elsewhere (27). Cells were removed by extraction with 0.1% Triton X-100 in PBS. After removal of the lysed cells, the matrix was incubated with 25 mmol/l NH_4SO_4 in PBS, washed with PBS, and kept sterile under PBS in the refrigerator until use. Wells containing matrix produced by SMCs under the two growth conditions were preincubated with binding buffer (10 mmol/l HEPES, pH 7.3, 20 mmol/l NaCl, 5 mmol/l Ca^{2+}, 2 mmol/l Mg^{2+}, 1 g/l BSA) for 30 min at 37°C. Then, ^{125}I-labeled LDL was added in binding buffer with or without an excess of cold LDL. After a 2-h incubation at 37°C, the matrix was washed 3 × 10 min with binding buffer and incubated for 30 min at 37°C with 0.1 ml 10 mmol/l Tris-HCl, pH 7.4, 0.5 mol/l NaCl, and 2 mmol/l EDTA. The released radioactivity was collected, and the wells were rinsed with another 0.1 ml of the same buffer. The remaining (tightly bound) ^{125}I-LDL was collected in 2 × 0.1 ml 0.2 mol/l NaOH. Radioactivity was measured in a gamma counter.

The total pool of proteoglycans produced by smooth muscle control cells and cells grown with 300 µmol/l linoleic acid in the culture medium were metabolically labeled by incubating with [^{35}S]sulfate, which incorporates into the glycosaminoglycan side chains on proteoglycans, and purified by ion exchange chromatography (28). Binding of the proteoglycan preparations to LDL was evaluated by Gel-Shift assay (29).

RESULTS

NEFA-induced alterations in ECM proteoglycans. To investigate the effect of NEFAs on the growth of arterial SMCs and on their synthesis of ECM proteins, we exposed the cells to NEFA and BSA in complete medium with FBS in sparse cultures. Under these conditions, the SMCs were proliferating and metabolically active. Exposure of arterial SMCs to 300 µmol/l linoleic acid decreased cell proliferation ~25% (not shown). Glucose uptake was increased in SMCs after exposure to linoleic acid. Insulin-stimulated glucose uptake was 666 ± 154 cpm/mg cell protein in the control cells and 927 ± 13 cpm/mg cell protein (n = 3) after exposure to 100 µmol/l linoleic acid for 48 h. The morphology of arterial SMCs exposed to 300 µmol/l linoleic acid was strikingly different from that of control cells (Fig. 1). No difference in morphology was seen after 4 h. The change in shape appeared gradually after 24–48 h, and the difference was more evident after longer culture in the presence of NEFAs. Whereas the control cells reached confluency after several days in culture, the SMCs that were cultured with NEFAs in the media got denser but did not reach confluency when kept in culture for up to 3

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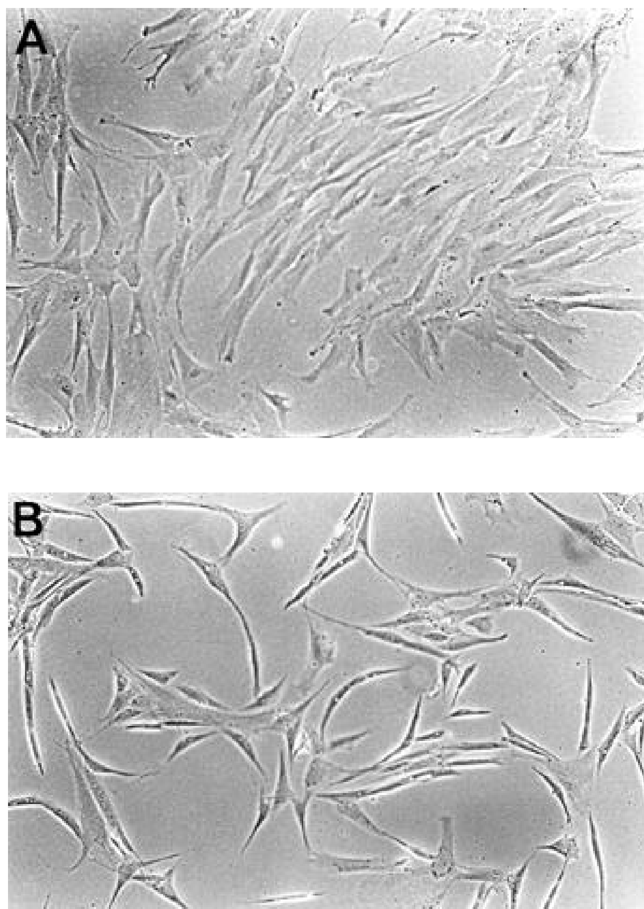

FIG. 1. Appearance of SMCs after exposure to linoleic acid. Human arterial SMCs were exposed to 100 µmol/l BSA in Eagle's minimum essential medium (EMES) 10% FBS (*A*) or to the same medium but with 300 µmol/l linoleic acid (*B*) for 96 h.

weeks. The change in cell shape appeared to be reversible. If the culture medium was returned to control medium, the cell shape returned to that of control cells within 24–48 h. Culturing the cells in 25 mmol/l glucose did not give the same change in cellular shape as that seen with the addition of NEFAs to the medium. Cell viability was similar with and without linoleic acid. Thus, Tryphan blue exclusion was similar in the wells with control cells grown with 100 µmol/l BSA (3.1 ± 1.6% blue cells, $n = 5$) and in the wells with cells grown with 100 µmol/l BSA and 300 µmol/l linoleic acid (2.0 ± 1.2% blue cells, $n = 5$).

To investigate the effect of NEFAs on the synthesis of ECM glycoproteins by the SMCs, we measured the relative production of mRNAs for several ECM proteins and some of the gene products. After incubating human arterial SMCs for 48 h with 100 and 300 µmol/l of linoleic acid, syndecan 4, decorin, and versican were upregulated in a dose-dependent way, while fibronectin and perlecan appeared to be unchanged or downregulated by the same treatment (Fig. 2). To explore whether the number of double bonds in the fatty acids, and by implication sensitivity to oxidation, was critical for the observed effect, the expression of fibronectin, versican, decorin, and G-3-PDH was measured in SMCs exposed to linoleic acid (18:2) or oleic acid (18:1). Both linoleic and

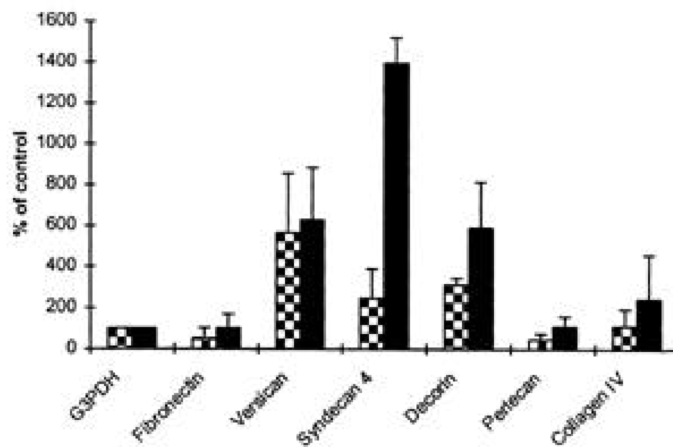

FIG. 2. Expression of ECM proteoglycans core proteins and ECM proteins after exposure of SMCs to linoleic acid. Human arterial SMCs were exposed to 100 µmol/l BSA in EMES 10% FBS without (control) or with 100 µmol/l (▚) or 300 µmol/l (■) linoleic acid for 48 h. Total RNA was prepared, and mRNAs for specific proteins were quantified. For comparison of the effects on mRNA expression between genes with different levels of expression, the mRNA data shown are corrected for content of mRNA for G-3-PDH in the respective total RNA preparation. For individual genes, the bars represent values above the expression for control cells. Values are means ± SD of four experiments for fibronectin, decorin, versican, and perlecan. For syndecan 4 and collagen IV, the bars represent the range for two experiments.

oleic acid upregulated the expression of versican and decorin to a similar extent (Fig. 3), indicating that extracellular free radical–mediated oxidation of NEFAs is an unlikely cause of the observed effects on protein expression. Separation of proteoglycans produced under the two growth conditions by SDS-PAGE and autoradiography indicated a difference in the distribution of proteoglycan sizes in the two samples (not shown). The decorin produced by arterial SMCs exposed to linoleic acid had a wider distribution of molecular weights with a shift toward larger molecules compared with that of control cells (Fig. 4). The change in size was

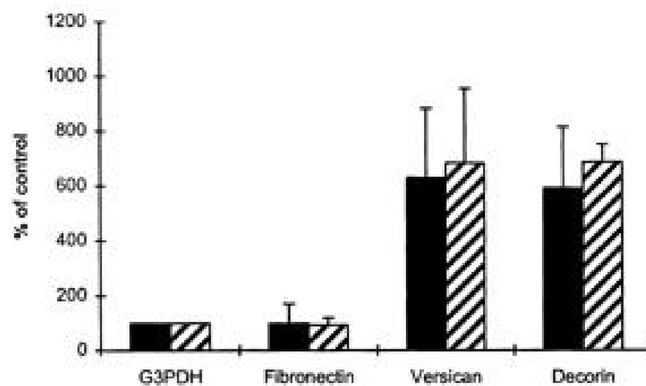

FIG. 3. Comparison of the effects of oleic and linoleic acid on the expression of fibronectin, versican, and decorin by SMCs. Human arterial SMCs were exposed to 100 µmol/l BSA in EMES 10% FCS without (control) or with 300 µmol/l linoleic acid (■) or oleic acid (▨) for 48 h. Total RNA was prepared, and mRNAs for fibronectin, versican, and decorin were determined. Data are presented as in Fig. 2. Error bars represent the average of four experiments ± SD for linoleic acid or two experiments ± range for oleic acid.

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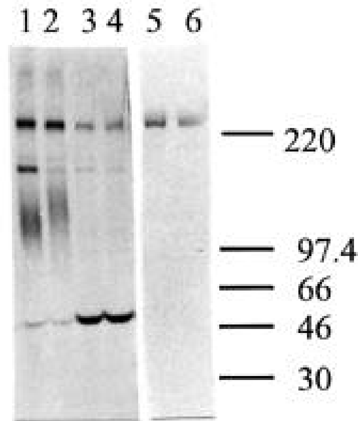

FIG. 4. Immunoprecipitation of decorin and fibronectin after exposure to linoleic acid. Human arterial SMCs were exposed to 100 μmol/l BSA in EMES 10% FBS without (control, *lanes 1, 3,* and *5*) or with 300 μmol/l linoleic acid (*lanes 2, 4,* and *6*) for 24 h. Then the media were changed to the same media with the addition of [^{35}S]methionine, and the cells were grown for an additional 72 h. Decorin (*lanes 1–4*) was immunoprecipitated from the media using a polyclonal anti-human decorin antibody and protein A-Sepharose before (*lanes 1* and *2*) and after (*lanes 3* and *4*) treatment with chondroitinase ABC and heparitinase. Fibronectin (*lanes 5* and *6*) was immunoprecipitated from the media with a monoclonal anti-human fibronectin antibody, rabbit anti-mouse secondary antibody, and protein A-Sepharose. After SDS-PAGE, the gel was dried and photographic film was exposed. *Lanes 1–4* (decorin) were exposed for 19 days. *Lanes 5* and *6* (fibronectin) were exposed for 3 h.

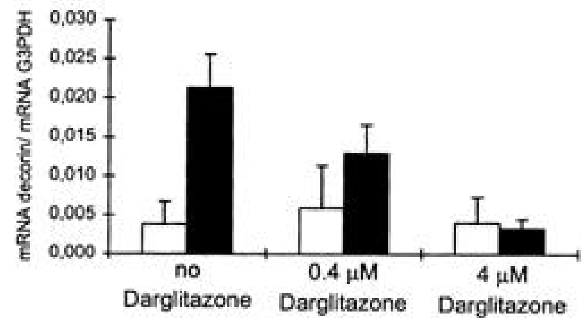

FIG. 6. Expression of decorin after exposure of SMCs to linoleic acid and darglitazone. Human arterial SMCs were exposed to 100 μmol/l BSA and 340 nmol/l insulin in EMES 10% FBS without (control, □) or with 300 μmol/l linoleic acid (■) for 48 h in the presence or absence of indicated concentrations of darglitazone. Total RNA was prepared, and mRNA for decorin and G-3-PDH were determined. Error bars represent the average of two experiments ± range.

caused by an increase in the carbohydrate component of decorin, since the size of the core protein did not change (Fig. 4). As decorin carries only one glycosaminoglycan chain per protein, the probable explanation is that the average length of the glycosaminoglycan chains increased. Versican and perlecan were also immunoprecipitated from culture media, but since these proteoglycans are too large to enter the polyacrylamide gel, it was difficult to evaluate changes in glycosaminoglycan size. The membrane-bound proteoglycan syndecan, produced by cells grown in the presence of linoleic acid, was estimated with an ELISA assay (Fig. 5). Relative to control cells, syndecan in the NEFA-treated cells was increased. There was no effect of NEFA on fibronectin production in the same cell preparations (Fig. 5).

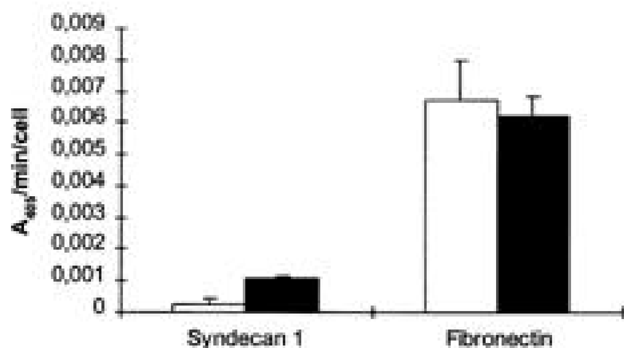

FIG. 5. Detection of syndecan 1 and fibronectin on cells. Human arterial SMCs were seeded sparsely in a 96-well plate. The next day, the media were changed to 100 μmol/l BSA in EMES 10% FBS without (control, □) or with 300 μmol/l linoleic acid (■), and the cells were grown an additional 72 h. Cells were then treated as described in METHODS to evaluate the content of syndecan 1 and fibronectin. Results were correlated for the number of cells that were counted in parallel lanes before fixation of the cells. Averages of four wells ± SD are shown.

To evaluate whether the effects of linoleic acid on expression of ECM proteins were influenced by the presence of ligands for the peroxisome proliferator-activated nuclear receptor (PPAR)-γ, darglitazone, a thiazolidinedione, was added to SMCs in the presence and absence of linoleic acid. Total RNA was prepared, and the expression of decorin and G-3-PDH (control) was determined (Fig. 6). Darglitazone appeared not to affect the expression of decorin in the absence of linoleic acid. However, in a dose-dependent manner, darglitazone diminished the induction of decorin expression caused by linoleic acid.

Changes induced by NEFAs on LDL interaction with matrix. To examine whether the alterations induced by NEFAs on the ECM structure caused changes in its capacity to interact with LDL, we measured their binding to ECM and to proteoglycans prepared from cells grown with or without NEFAs. The basement membrane from SMCs grown in low (only the NEFAs present in 10% FBS) or high (300 μmol/l) linoleic acid concentrations was used to evaluate the binding of LDL. In this experiment, cells were seeded in 48-well plates that had been coated with collagen I. Cells were cultured for 5 days. By then, the control cells were confluent, whereas the cells grown in 300 μmol/l linoleic acid were nonconfluent and appeared to have somewhat altered morphology, as shown in Fig. 1. The cell number was 50% lower in the wells in which cells were grown with 300 μmol/l linoleic acid in the medium than in the control wells. There was a small but significant increase in the amount of ^{125}I-LDL releasable by mild salt treatment from the matrix of cells grown for 5 days with linoleic acid as compared with control cells when incubated with 5 and 20 μg/ml LDL (not shown). More dramatically, the capacity to bind ^{125}I-LDL tightly (released with 0.2 mol/l NaOH) increased severalfold when the cells were grown with linoleic acid (Fig. 7*A*).

Metabolically ([^{35}S]sulfate) labeled proteoglycans were also isolated from control cells and from cells cultured with 300 μmol/l linoleic acid in the medium. The labeled proteoglycans were used for evaluation of binding parameters using increasing amounts of unlabeled LDL and electrophoretic

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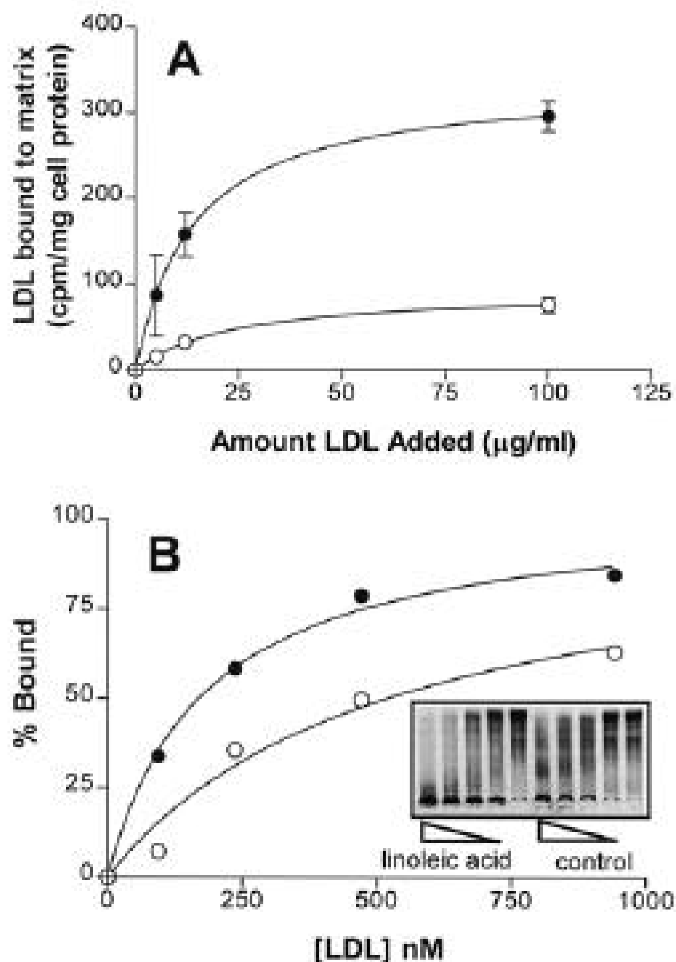

FIG. 7. LDL binding to ECM and to proteoglycans. ***A*****: Human arterial SMCs were exposed to 100 μmol/l BSA in EMES 10% FBS with or without 300 μmol/l linoleic acid for 5 days. Matrix was prepared as described in METHODS. Wells were incubated with 5, 20, or 100 μg/ml ^{125}I-LDL. Tightly bound ^{125}I-LDL, released after incubation with 0.2 mol/l NaOH, is shown. Binding in the presence of 1 mg/ml unlabeled LDL was subtracted to correct for unspecific binding. Error bars represent SD (n = 3) when larger than symbol.** ***B*****: Proteoglycans were metabolically labeled with [^{35}S]sulfate and isolated from cells cultured under the two conditions, and 40,000 cpm of each preparation was mixed with 0, 50, 125, 250, or 500 μg/ml LDL. The binding was evaluated by Gel-Shift. The result shown is representative of three experiments. ●, with fatty acid; ○, without fatty acid.**

band shift analysis. This method allows one to measure interactions at physiological ionic conditions (29). The titration curves allowed us to measure the dissociation constants, K_D (Fig. 7*B*). The K_D for the affinity of LDL for the proteoglycan preparation from control cells was 522 nmol/l, and the K_D for the proteoglycan preparation from the cells grown with 300 μmol/l linoleic acid in the medium was 180 nmol/l, indicating an almost threefold higher affinity of LDL for the proteoglycans from NEFA-treated cells. The total binding was not different (100 vs. 104 nmol).

DISCUSSION

The biochemical basis of the association between type 2 diabetes and cardiovascular disease remains to be characterized (30). Certainly the dyslipidemia of type 2 diabetes can be a contributor to this association, but several authors believe that a basic abnormality of the vascular system may also exist (31). A permanent oversupply of NEFAs to cells of the arterial wall may be one factor connecting dyslipidemia with tissue-related abnormalities contributing to atherogenesis (30). NEFAs appear to increase endothelium basement membrane permeability (14). This can contribute to atherogenesis by increasing the influx of lipoproteins to the arterial intima. Our findings on the overproduction of ECM proteoglycans by SMCs, if present in vivo, could also be atherogenic by inducing a matrix with higher affinity for apoB-containing lipoproteins. In the arterial intima, the nature of the proteoglycans and size of their glycosaminoglycan chains appear to modulate the retention of LDL (32–35). Our results suggest that, at least in vitro, LDL has a higher affinity for ECM synthesized by SMCs incubated with high levels of NEFAs. Increased endothelial permeability to LDL and its increased retention by ECM can act synergistically as contributors to atherosclerotic plaque development. However, this hypothesis needs further documentation in an in vivo model.

The levels of NEFAs in this study, 100 and 300 μmol/l, were selected to represent moderate to high levels of FFAs. The amount of albumin used, 100 μmol/l, plus the albumin present in 10% FBS (35–40 μmol/l) should be close to that in the extracellular fluid of the intima (36). Plasma FFA concentrations in humans can range from 180 to 1,650 μmol/l (37). Essentially all NEFAs should be bound to albumin at the concentrations of FBS and albumin used here (18). We found no evidence of decreased cell viability in the conditions used. Nor could we detect free radical–mediated oxidation of the NEFAs in the media. The increase in glucose uptake observed after exposure to linoleic acid also indicates that the cells are metabolically active and viable. In addition, oleic acid, which is much less susceptible to free radical–mediated oxidation, seems to induce effects similar to those observed with linoleic acid. Therefore, we believe that extracellular oxidation products are not involved in the changes in ECM observed with linoleic acid.

The architecture and function of the intima of small and large arteries depend on the synthesis and remodeling of ECM components secreted by the endothelial cells and the SMCs of the media. Endothelial cells and their basement membrane constitute the first semipermeable barrier to separate circulating blood from the arterial wall. However, most of the volume in intima media is occupied by proteoglycans, collagens, and elastin secreted by the SMCs (12). Microangiopathies and macroangiopathies that occur as diabetic complications involve abnormalities of the extracellular intima. Some of the described alterations in microangiopathies are capillary basement membrane thickening and increased permeability (38,39). These alterations are associated with a decrease in the content of heparan sulfate proteoglycans. In patients with type 2 diabetes, the extracellular intima of large arteries have a decreased relative content of heparan sulfate proteoglycans and an increased relative content of dermatan sulfate proteoglycans (3). Long-term exposure to hyperglycemia was postulated to be one of the agents that induces changes in the ECM collagens and proteoglycans of endothelial cells (40). However, Hennig and colleagues (13,14,41–43) found that in vitro exposure to high levels of NEFAs alters the permeability of the endothelium. Oleic and linoleic acids were more potent than saturated fatty acids in their effect on

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permeability. They also found that NEFAs decreased heparan sulfate proteoglycan synthesis but increased that of an uncharacterized chondroitin sulfate proteoglycan. They concluded that the observed alterations in permeability were related to the changes in ECM proteoglycan composition and consequently ECM structure (13,14).

Retention of apoB-containing lipoproteins in the ECM produced by human arterial SMCs and subsequent modification of the lipoproteins have been proposed to be important initial steps in the development of atherosclerosis (7,8). Our results indicate that substantial alterations of cell morphology and ECM proteoglycans can be induced by incubation of human arterial SMCs with moderate concentrations of NEFAs. The changes in cell morphology observed may be related to the appreciable increase in the expression of the genes for the core protein of syndecan, decorin, and versican and synthesis of their gene products. Syndecans are pericellular chondroitin sulfate/dermatan sulfate proteoglycans involved in cell matrix association. Syndecan 4 is a primary response gene in vascular SMCs (44), and it is enriched in focal adhesions (45). Decorin is an extracellular proteoglycan that participates in collagen bundle organization. Versican, on the other hand, is a large proteoglycan of the ECM and the main proteoglycan of the arterial intima (12). NEFAs, in addition, increased the apparent size of the glycosaminoglycan moiety of decorin. These results allow us to speculate that in vivo chronic exposure to moderately elevated NEFAs, as in type 2 diabetes, may contribute to excess production of altered ECM in small and large vessels (30). This possibility, however, needs to be explored in an in vivo model of chronic exposure to high circulating NEFAs that could allow analysis of the intima ECM of arteries.

The list of genes associated with carbohydrate and lipid metabolism whose expressions are controlled by the intracellular levels of NEFA is growing (46–49). In addition, the infusion of NEFAs in rats causes an increase in the flux of glucose into the hexosamine pathway (7). The final products of this pathway are UDP-GlcNAc and UDP-GalNAc, which are building blocks for the synthesis of glycosaminoglycans and proteoglycans. This suggests a mechanism by which increased substrate availability could cause the arterial SMCs in our experiments to increase their production of syndecan and decorin when exposed to high NEFAs. However, this does not explain why an increase in the production of other proteoglycans, such as perlecan, was not detected. Turnover studies may clarify this discrepancy. Many of the effects of NEFAs on gene expression appear to be mediated by PPAR-γ and PPAR-α. PPAR-γ is expressed in many cells of mesodermic origin, including adipocytes, fibroblasts, and macrophages. PPAR-α is present in hepatocytes (50). Recently, PPAR-γ was found in rat arterial SMCs, but its function there remains to be studied (51). PPAR-γ in adipocytes increases triglyceride synthesis using the intracellular pool of fatty acyl-CoA. If PPAR-γ does the same in SMCs, it may limit the actions of fatty acyl-CoA on glucose utilization and lower the contribution of the hexosamine pathway. This action may explain why, in our model, darglitazone inhibits the effects of NEFA in a dose-dependent manner (Fig. 7). PPARs are promiscuous, and their response to different ligands may be diverse (50). Thus, darglitazone, which has a high affinity for PPAR-γ, may be competing with the fatty acid for PPAR-γ but not inducing the same type of response as NEFAs. Experiments with other ligands for PPARs could help to clarify their function in the metabolism of SMCs. In summary, our results indicate that moderately elevated NEFA concentrations modulate the production of pericellular and ECM macromolecules that are important contributors to the structure and function of the arterial intima. A chronic alteration of these properties may contribute to the vascular abnormalities present in type 2 diabetes and potentiate the atherogenicity of LDLs.

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