Rodríguez-Lee M et al

Fatty acids cause alterations of human arterial smooth muscle cell proteoglycans that increase the affinity for LDL

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 H₂O 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-µm filters and the final fatty acid concentration was measured (NEFA-C kit from Wako). Agarose gel electrophoresis of the final solutions containing 10⁵ cpm/µl of ¹⁴C-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% O₂, 5% CO₂, 74% N₂) in M199 supplemented with 10% (v/v) human serum, 100 U/ml penicillin, 100 µg/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 \( \mu \text{mol/l} \) 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 \( \mu \text{mol/l} \) were used. When added, the PPAR\( \gamma \) 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 \( \mu \text{Ci/ml} \) \([^{35}S]\) Sulfate and 10 \( \mu \text{Ci/ml} \) D-[6-\( ^3 \)H] 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-\( \mu \text{m} \) filter and the total cell protein was determined with the Modified Lowry Protein Assay Kit (Pierce Biotechnology). Cold carrier (100\( \mu \text{g} \) 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\( ^\circ \)C. GAGs were then isolated by precipitation with 95% ethanol and characterized with agarose gel electrophoresis and chondroitinase treatment as described.\(^3\) Evaluation of the length of isolated GAG chains was made by polyacrylamide gel electrophoresis (PAGE).\(^4\) Aliquots containing equal amounts of incorporated D-[6-\( ^3 \)H] 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\( ^\circ \)C for 10 min, 48\( ^\circ \)C for 30 min, and 95\( ^\circ \)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 AAGGCCACTATCATCCTCTCTTCT / AAAGTCAAATAAGCCTCTCTGTGAAA and probe TTGCACAAGTTTCTCGTGAGACC; perlecan TGGCTGACAGCATCTCAGGA / CGATGGAGCGAGTGAGTGGAAGATTTGAGACAGCCCTG and versican ACGATGTGTATTTATTTGTGGATCTCAGGA / AGCCTCCTCGAAGGTGAATTGAATT and probe TGATGTGTTCACTCAGCTGCCCA. 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 $^{35}$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 ($\text{app}K_D$) 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:


