Platelet-derived Growth Factor Differentially Regulates the Expression and Post-translational Modification of Versican by Arterial Smooth Muscle Cells through Distinct Protein Kinase C and Extracellular Signal-regulated Kinase Pathways

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The synthesis of proteoglycans involves steps that regulate both protein and glycosaminoglycan (GAG) synthesis, but it is unclear whether these two pathways are regulated by the same or different signaling pathways. We therefore investigated signaling pathways involved in platelet-derived growth factor (PDGF)-mediated increases in versican core protein and GAG chain synthesis in arterial smooth muscle cells (ASMCs). PDGF treatment of ASMCs resulted in increased versican core protein synthesis and elongation of GAG chains attached to the versican core protein. The effects of PDGF on versican mRNA were blocked by inhibiting either protein kinase C (PKC) or the ERK pathways, whereas the GAG elongation effect of PDGF was blocked by PKC inhibition but not by ERK inhibition. Interestingly, blocking protein synthesis in the presence of cycloheximide abolished the PDGF effect, but not in the presence of cycloheximide, indicating that GAG synthesis that results from PKC activation is independent from de novo protein synthesis. PDGF also stimulated an increase in the chondroitin-6-sulfate to chondroitin-4-sulfate ratio of GAG chains on versican, and this effect was blocked by PKC inhibitors. These data show that PKC activation is sufficient to cause GAG chain elongation, but both PKC and ERK activation are required for versican mRNA core protein expression. These results indicate that different signaling pathways control different aspects of PDGF-stimulated versican biosynthesis by ASMCs. These data will be useful in designing strategies to interfere with the synthesis of this proteoglycan in various disease states.

Proteoglycans are extracellular matrix macromolecules that are composed of a core protein to which are attached covalently linked glycosaminoglycan (GAG) chains that can vary in number and size depending on the nature of the core protein (1). The biosynthesis of these complex macromolecules involves pathways that regulate both core protein synthesis and post-translational processing of the GAG chains (2–5). At present, it is not clear whether similar or different signaling pathways control both of these aspects of proteoglycan biosynthesis. Because proteoglycan accumulation in the extracellular matrix contributes to many disease states, including vascular diseases (6–9), it becomes critical to further define the key signaling pathways that regulate the different aspects of the biosynthesis of these macromolecules. Such studies will be important in the future to further define therapeutic targets for the prevention of cardiovascular and other diseases (10, 11).

Platelet-derived growth factor (PDGF) is a mitogen that has been implicated in cardiovascular disease and has been shown to have a number of effects on arterial smooth muscle cells (ASMCs), such as stimulation of proliferation, migration, and extracellular matrix secretion (12). PDGF stimulates expression of the large chondroitin sulfate (CS) proteoglycan, versican; it mediates elongation of the CS chains attached to the core protein of versican; and it increases the 6:4 sulfation ratio on the monosulfated disaccharides of CS (13–16). The signaling pathways that control these different aspects of proteoglycan biosynthesis by ASMCs are not understood.

PDGF activates a number of signaling pathways in ASMCs, including those that involve extracellular signal-regulated kinase (ERK; one of the mitogen-activated protein kinases) and several protein kinase C (PKC) isoforms that influence the proliferative and migratory activity of ASMCs (17). We now report that PDGF stimulation of versican gene expression is mediated via PKC- and ERK-dependent pathways in nonhuman primate ASMCs, whereas PDGF-mediated elongation of CS chains

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3. The abbreviations used are: GAG, glycosaminoglycan; PDGF, platelet-derived growth factor; ASMC, arterial smooth muscle cell; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; CS, chondroitin sulfate; CPC, cetyl pyridinium chloride; TPA, 12-O-tetradecanoylphorbol-13-acetate; MAP, mitogen-activated protein; M KK, MAP kinase; NBS, newborn calf serum.
attached to the core protein of versican is mediated via PKC but not ERK. In addition, PDGF modification of the sulfation pattern assessed as an increase in the chondroitin-6-sulfate to chondroitin-4-sulfate ratio was dependent on PKC signaling. These results indicate that PDGF acts through distinct and different signaling pathways to regulate different aspects of versican biosynthesis in ASMCs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Guandine HCl (grade 1), Tris base, cetyl pyridinium chloride (CPC), urea, bovine serum albumin, blue dextran, H7, HA1004, 12-0-tetradecanoylphorbol-13-acetate (TPA), and methyl-β-D-xlyopyranoside (xylloside) were purchased from Sigma-Aldrich; 6-aminohepxanic acid, benzamidine HCl, NTB-2 autoradiography emulsion, and XAR-2 film were from Eastman Kodak Co. (Rochester, NY); chondroitin ABC lyase was from Seikagaku Kogyo Co., Ltd., through MP Biomedicals (Costa Mesa, CA); Triton X-100 was from Roche Applied Sciences; Sepharose CL-2B and Sepharose CL-6B were from GE Healthcare; electrophoresis chemicals were purchased from Bio-Rad; Na[32P]SO4 (43 Ci/mg S; carrier-free) and Universal scintillation fluid were from MP Biomedicals; GF109203X and PD098059 were from Enzo Life Sciences (Plymouth Meeting, MA); DEAE-Sephalac was from Sigma-Aldrich; and Aquacide II was from EMD Chemicals. Enlightening rapid autoradiography enhancer was from Dupont; Zeta-Probe GT blotting membranes were from Bio-Rad; full-length human biglycan cDNA (P16) and full-length bovine decorin cDNA were provided by Dr. L. Fisher (18) and Dr. M. Young (19), respectively, of the National Institute of Dental and Craniofacial Research, NIH (Bethesda, MD); and human versican cDNA (C7) was from Dr. E.Ruoslhanti (20) of the Burnham Institute for Medical Research (Santa Barbara, CA). 5'-O-[α-32P]dCTP was from GE Healthcare. PDGF-AB, purified from human platelets, was a gift from Elaine W. Raines (University of Washington). This preparation was used for all experiments unless otherwise noted. Recombinant human PDGF-AA, -BB, and -AB from PeproTech Inc. (Rocky Hill, NJ) were used in experiments designed to compare the effects of those three isoforms on versican expression and GAG synthesis. All of the other chemicals were reagent grade.

**Cell Culture: General Procedure**—ASMC cultures were established from strips of intimal-medial tissue from the thoracic aorta of 3–4-year old pigtail monkeys (Macaca nemestrina). The cells were cultured as previously described (21–23). ASMCs from passages 4–9 were seeded at a density of 6 × 10⁵ cells/100-mm plastic culture dish and cultured for 7 days in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 5% newborn calf serum (NBS) (Intergen, Purchase, NY), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids containing 100 µg/ml streptomycyn (Invitrogen) and 100 units/ml penicillin G. The cells were kept at 37 °C in a humidified atmosphere of 5% CO₂ in air. After reaching visual confluence, the cells were growth-arrested by starvation for 48 h in low serum medium (Dulbecco’s modified Eagle’s medium supplemented with 0.1% NBS). The medium was then changed, fresh starvation medium was added with or without inhibitors, and stimulators were added to the appropriate culture dishes 30 or 60 min later (τ = 0). After different incubation periods, the cells were metabolically labeled with 100 µCi/ml [35S]sulfate for 6 h, and then the media and cell layer extracts were harvested and submitted to the analyses described below.

**Stimulation and Metabolic Labeling**—The medium was replaced by fresh medium containing 0.1% NBS, with or without additional factors, 24 h prior to harvest. The concentrations of PDGF (10 ng/ml) and TPA (5 nM) were determined empirically by assessing the increase in incorporation of [35S]sulfate into proteoglycans by ASMCs exposed to these factors. The cells were labeled with 50 µCi/ml [35S]sulfate in complete medium for 6–12 h following the addition of PDGF. For desaccharide analysis, the cells were labeled with 50 µCi/ml of [35S]sulfate between 18 and 24 h. For each experiment, the number of cells was determined with a particle counter after trypsinization of parallel dishes.

PKC activity was inhibited by H7 (50 µM). A negative control, consisting of the structural analog, HA1004, was used at this same concentration to examine the possibility of nonspecific effects caused by inhibition of other protein kinases (24). The pan-PKC inhibitor GF109203X (5 µM) was used to further implicate a role for PKC. PD098059 (30 µM) was used to inhibit the ERK pathway. We have previously shown that this concentration of PD098059 inhibits PDGF-induced activation of mitogen-activated protein kinase kinase (MKK) and the downstream ERK in ASMCs (17). The inhibitors were not cytotoxic at the concentrations used, as determined by a lactate dehydrogenase assay (Roche Applied Science) and by visual inspection (lack of altered morphology and floating cells).

To further evaluate whether differences existed in the regulation of core protein versus GAG synthesis, some cultures were treated with cycloheximide in the presence or absence of xyloside. Cycloheximide was used at 1 µg/ml in 0.01% DMSO (dimethyl sulfoxide), and xyloside was used at 0.5 mM and added 24 h before PDGF or TPA. [35S]Sulfate labeling occurred at 6 h after growth factor addition, and media were harvested at 12 h after stimulation.

**Measurement of Sulfate Incorporation into Proteoglycans**—Proteoglycans were extracted from the medium (22, 23). The incorporation of [35S]sulfate into proteoglycans was measured by CPC precipitation (25). Briefly, aliquots of the medium were spotted on filter paper and washed five times for 1 h in 1% CPC containing 0.05 M NaCl. The amount of precipitate on the dried filter paper was determined by liquid scintillation counting.

**Proteoglycan Isolation and Analysis**—Radiolabeled medium samples were directly applied to DEAE-Sephalac minicolumns (1 ml of resin) equilibrated with 8 mM urea, 2 mM EDTA, 0.5% Triton X-100, 50 mM Tris-HCl. The columns were washed extensively with the same buffer containing 0.25 M NaCl to remove glycoproteins and free, nonincorporated [35S]sulfate, and the proteoglycans were eluted with the same buffer containing 3 M NaCl. For further analyses, concentrated preparations of purified proteoglycans were precipitated in 3.5 volumes of 95% ethanol containing 1.5% potassium acetate and 50 µg/ml carrier chondroitin sulfate.

Chromatography on Sepharose CL-2B was used to fractionate versican from the small chondroitin/dermatan sulfate proteoglycans (13, 15). Approximately 1 ml of concentrated total
medium proteoglycans from the DEAE-Sephacel chromatography were applied to columns (1 × 105-cm gel bed) equilibrated in 50 mM Tris buffer, pH 7.5, containing 8 M urea, 2 mM EDTA, and 0.5% Triton X-100. The column was eluted at a flow rate of 3.5 ml/h, and fractions of 1 ml were collected. The void volume of the columns was determined by the elution of tritium-labeled DNA from Escherichia coli. The elution position of free $[^{35}S]$ was used as a marker for the total volume.

Chemical Cleavage and Analysis of GAG Chains—GAGs were chemically released from proteoglycans by reductive $\beta$-elimination (26). The alkaline $\beta$-elimination was done with 1 M sodium borohydride in 50 mM NaOH for 24 h at 45 °C. The reaction was terminated by neutralizing the sample with glacial acetic acid. GAGs prepared by reductive $\beta$-elimination were chromatographed on a Sepharose CL-6B column (0.7 × 63 cm) in 0.2 M Tris-HCl, pH 7.0, 0.2 M NaCl (27). Determination of the relative proportions of 6- and 4-sulfated disaccharide was carried out by descending paper chromatography (28) of chondroitin ABC lyase digests of isolated proteoglycan subclasses (13).

SDS-PAGE—SDS-PAGE was performed in a 4–12% gradient resolving gel with a 3% stacking gel (29). After the run, the gel was treated with Enlightening rapid autoradiography enhancer for 30 min and dried, and the labeled proteoglycans were visualized by exposing the dried gel to a Kodak XAR-2 film at −70 °C. The appropriate molecular weight standards (Invitrogen) were run in separate lanes.

Isolation and Northern Blotting of RNA—Total RNA was prepared by the single-step method as described by Chomczynski and Sacchi (30). Ribonucleic acids (10–15 μg/lane) were electroforesed overnight in 1% (w/v) agarose gels containing formaldehyde (31), transferred to Zeta-Probe GT blotting membranes, and cross-linked with UV light for Northern blotting and hybridization with $^{32}$P-labeled cDNA probes, as previously described (32). Several cDNAs were used as probes for proteoglycan mRNA on Northern blots, including full-length human biglycan cDNA (P16), full-length bovine decorin cDNA and human versican cDNA clone (C7) (33), and our cloned isoform V3. Northern blots were normalized for loading by comparison with ethidium bromide staining of bovine 28 S rRNA cDNA. The probes were $^{32}$P-labeled by random priming, using 5′-α-32PdCTP and hybridized with RNA on Northern blots prepared as described above. The autoradiograms were digitized on a flat bed scanner and imported into the National Institutes of Health Image software for quantitation.

RESULTS

The Stimulatory Effects of PDGF on Proteoglycan Accumulation Are Mediated by PKC and ERK Activation—The levels of expression of proteoglycan core proteins and post-translational modifications in the GAG chains are reflected by alterations in the extent of incorporation of $[^{35}S]$sulfate. To explore whether similar signaling pathways were involved in both core protein and GAG synthesis, we used an activator and several inhibitors to assess the role of protein kinases in this response. The phorbol ester TPA activates the classical and novel PKC isoforms, whereas compounds H7 and GF 169203X block PKC and cAMP (protein kinase A) activity. HA1004 is an H7 structural analog with a 20-fold lower inhibitory potency toward PKC but with similar effects on other kinases (34). Compounds H7 and HA1004 have been used together previously to define the role of PKC in cellular processes (35, 36). All of the compounds were not overly cell toxic because they did not increase lactate dehydrogenase release at the concentrations used in these experiments (data not shown). Visual inspection of the cells revealed no altered morphology or floating cells. PDGF (10 ng/ml) treatment resulted in a 3–4-fold increase in total $[^{35}S]$sulfate incorporation above the low serum control (Fig. 1A). We chose the AB isoform of PDGF because we had used this isoform in our previous studies to demonstrate a PDGF effect on proteoglycan synthesis in these ASMCs (13–15, 37). We also have shown that ASMCs contain 10-fold more receptors for PDGF-BB than for PDGF-AA (38). To address whether the $\alpha$ or $\beta$ receptors are required for regulation of versican core protein synthesis by PDGF, we analyzed whether differences could be detected in the versican response to PDGF-AA, -BB, and -AB. All three isoforms stimulated mRNA expression of the versican protein, indicating that signaling was regulated through both the $\alpha$ and $\beta$ receptors (supplemental Fig. S1).

The phorbol ester, TPA, stimulated $[^{35}S]$sulfate incorporation but to a lesser extent (60%) compared with PDGF (Fig. 1A). As expected, the response to TPA was prevented by treatment with H7 (Fig. 1A). The stimulatory effect of PDGF treatment on $[^{35}S]$sulfate incorporation into secreted proteoglycans was totally abolished by treatment of cells with H7 and was unaffected or marginally increased by HA1004. These data indicate that the stimulatory effect of PDGF on total $[^{35}S]$sulfate incorporation is mediated via a PKC-dependent pathway. PDGF also activates ERK signaling in ASMCs (17). We evaluated the effect of PD098059, a selective cell-permeable inhibitor that binds to inactive MKK 1/2, preventing its phosphorylation by Raf and thus selectively inhibiting the MAP kinase ERK 1/2 but not p38 or JNK MAP kinases (39). The stimulatory effect of the PKC activator TPA on $[^{35}S]$sulfate incorporation was not affected by PD098059, indicating that the ERK pathway is not downstream of PKC (Fig. 1B). PD098059 had no effect on basal incorpora-
tion of [35S]sulfate into secreted proteoglycans but inhibited the effect of PDGF by 50% (Fig. 1B).

The Stimulatory Effects of PDGF on Specific Proteoglycans Are Differentially Regulated by PKC and ERK Activation and Independent of Protein Kinase A—Monkey ASMCs labeled with [35S]sulfate secrete two predominant peaks of radioactivity separated on Sepharose CL-2B size exclusion columns, and these peaks have previously been identified as the large molecular weight CS proteoglycan versican and the lower Mr peak containing the dermatan sulfate proteoglycans biglycan and decorin (13, 14, 22). Using 6-h labeling periods between 0 and 30 h, PDGF stimulates an increase in the proportion of the radioactivity associated with the high molecular weight peak, versican, beginning at 12 h following stimulation and continuing through the entire labeling period (Fig. 2). On the other hand, no changes in the amount of radioactivity in the second peak occurred at any of the 6-h labeling times. However, both radiolabeled peaks shifted toward the Vo of the column, indicating that the proteoglycans in both peaks are hydrodynamically larger in the presence of PDGF (Fig. 2).

We evaluated the role of PKC and ERK in PDGF-mediated changes in the size and amounts of the two major radiolabeled proteoglycan populations, as assessed by molecular sieve chromatography on Sepharose CL-2B under dissociative conditions (13–15). PDGF caused a clear increase in the size of the versican peak, which was effectively blocked when the ASMCs were treated with H7 but not with HA 1004 (Fig. 3). Activation of PKC with TPA increased the molecular size of both proteoglycan peaks but did not increase the area under the curve of the versican peak (Fig. 3) when compared with control cultures (Fig. 3). The increased proteoglycan sizes induced by TPA were blocked by H7, but not HA1004, and H7 also reduced the amount of radioactivity of the versican peak relative to the basal level (Fig. 3). The MKK inhibitor PD098059 did not block the leftward shift in location of the peaks induced by PDGF but partially prevented the versican peak from increasing in size (Fig. 3).

PDGF Stimulates Versican mRNA Synthesis through PKC-dependent and ERK-dependent Signaling Pathways—PDGF treatment of ASMCs caused a marked increase in the level of secreted versican in these cultures (Fig. 2). We investigated the level of versican mRNA as a potential pathway through which PDGF regulates versican expression and secretion. PDGF treatment resulted in a 4.5-fold increase in versican mRNA, confirming our earlier finding (15) (Fig. 4). PKC stimulation by TPA resulted in a smaller stimulatory effect. PDGF-stimulated versican mRNA expression was abolished by the PKC inhibitors, H7 and GF 109203x (Fig. 4). These results support the observations in Fig. 2, suggesting that the increases observed in

**FIGURE 2.** Molecular sieve chromatography of newly synthesized proteoglycans following a time course of PDGF stimulation. ASMCs were stimulated with PDGF (10 ng/ml) and pulse-labeled with [35S]sulfate for 6-h periods from 0 to 30 h. Radiolabeled culture medium samples were chromatographed on Sepharose CL-2B molecular sieve columns under dissociative conditions (8 M urea). The columns were loaded on the basis of equal cell numbers. The experiment was repeated two times with very similar results.

**FIGURE 3.** Molecular sieve chromatography of newly synthesized proteoglycans following PDGF stimulation in the presence of specific kinase inhibitors. ASMCs were stimulated with PDGF (10 ng/ml) or TPA (5 nM) for 24 h under control conditions or in the presence of H7 (50 μM), HA1004 (50 μM), or PD098059 (30 μM). Radiolabeled culture medium samples were chromatographed on Sepharose CL-2B molecular sieve columns under dissociative conditions (8 M urea). The experiment was repeated two times with very similar results.
incorporation of $[^{35}S]$sulfate were partially due to enhanced versican protein synthesis.

We have previously demonstrated that the expression of versican in these cells is genistein-sensitive (15) and thus thought to be linked via the PDGF signaling pathway most closely associated with the mitogenic action of PDGF (40). We therefore investigated the effect of inhibiting ERK activation on PDGF-mediated versican mRNA expression. PD098059 had no effect on the basal level of versican mRNA but markedly reduced the response to PDGF (Fig. 4B). Thus, the increase in mRNA for versican in response to PDGF in ASMCs is dependent on both PKC and ERK.

**The Stimulatory Effects of PDGF on Versican CS Chain Elongation and Composition Are Mediated by a PKC-dependent Pathway**—We assessed the contribution of changes in GAG chain size as the most likely determinant of changes in molecular size of the proteoglycans. ASMCs were treated for 24 h with PDGF in the presence of the inhibitors, and the proteoglycans were isolated from the culture medium and characterized. The versican peak was isolated from Sepharose CL-2B columns, and the GAG chains were chemically cleaved and analyzed by Sepharose CL-6B size exclusion chromatography (Fig. 5). Compared with control, PDGF treatment of cells resulted in larger GAG chains attached to the core protein of versican (Fig. 5A). This confirmed that the effect of PDGF treatment to increase proteo-
glycan size (Figs. 2 and 3) was due to an increase in GAG chain size. We also wished to determine whether the AA or BB isoforms of PDGF could cause these size shifts and whether the α receptor was involved. Therefore, GAG chains were isolated from versican after stimulation of ASMCs with each of the PDGF isoforms AA, BB, and AB and sized by molecular sieve chromatography on Sepharose CL 6B. Increased GAG size was observed after treatment with each of the isoforms, with the greatest effect occurring in response to PDGF-BB (Supplemental Fig. S2), indicating involvement of PDGFR-α and -β. Thus, signaling from both receptors contributes to pathways regulating versican core protein synthesis and GAG chain size.

Isolated GAG chains from cells treated with TPA were also larger than control, but to a lesser extent compared with the effect of PDGF (Fig. 5A). The effect of both PDGF and TPA to increase GAG chain size on proteoglycans was prevented by H7 (Fig. 5B). Treatment with HA1004 did not inhibit the effect of PDGF or TPA (Fig. 5C). These data indicate that PKC is involved in PDGF-mediated GAG chain elongation on the versican core protein. Notably, inhibition of ERK activation reduced PDGF-stimulated radiosulfate incorporation by 50% (Fig. 1). This decrease could be due to inhibition of PDGF-mediated core protein expression and/or GAG chain elongation. However, GAG chains cleaved from versican after blocking ERK activation by treatment with PD098059 in the presence of PDGF showed no decrease in size compared with PDGF treatment alone (Fig. 5D). This suggests a direct effect on core protein expression rather than on GAG chain elongation. In fact, because versican mRNA expression depends on ERK activation (Fig. 4B), the decrease in radiosulfate incorporation following ERK inhibition (Fig. 1) was due to fewer proteoglycan monomers, which, however, had normal sized GAG chains.

A further post-translational modification in GAG synthesis and structure induced by PDGF is an enrichment in sulfate at the 6 position of the GalNAc residue of the GlcA:GalNAc disaccharides that constitute the CS chains of versican. The signaling pathway through which PDGF regulates the selectivity of chondroitin-4- and chondroitin-6-sulfation is unknown. We used 6-h intervals to investigate the time course for the increase in the chondroitin-6-sulfate to chondroitin-4-sulfate ratio that occurs following PDGF stimulation of these ASMCs. No differences were seen at 6 h of labeling, but then an approximately linear increase in the ratio occurred through 30 h (Fig. 6A). This mimics the pattern of GAG chain elongation following PDGF stimulation (Fig. 2). This PDGF-mediated change in the chondroitin-6-sulfate to chondroitin-4-sulfate ratio was inhibited by H7 and unaffected by HA1004 (Fig. 6B). In contrast to the ability of the PKC activator TPA to activate GAG chain elongation in these cells (Figs. 3 and 5A), TPA did not change the chondroitin-6-sulfate to chondroitin-4-sulfate ratio when compared with unstimulated controls (Fig. 6B). These results indicate that PDGF utilizes PKC signaling to mediate changes in the sulfation pattern, but PKC activity alone is insufficient to generate this response.

The smaller dermatan sulfate proteoglycans, biglycan and decorin, can be separated by molecular sieve chromatography (Fig. 2). With some limitations, electrophoretic mobility can be used as an indicator of molecular size. PDGF and TPA (Fig. 7,
means rather involves activation of the GAG synthetic machinery. The data show the results from PKC activation does not involve PDGF. These experiments indicate that enhanced chain elongation that cycloheximide restored the increase in sulfate incorporation in the presence PDGF effect on sulfate incorporation, but added xyloside in the presence of tion into CPC precipitable material following PDGF treatment in the presence of PDGF; cycloheximide, a protein synthesis inhibitor; and arrested ASMCs were treated with various combinations of protein synthesis from GAG chain elongation. Thus, growth-arrested ASMCs were treated with various combinations of PDGF; cycloheximide, a protein synthesis inhibitor; and methyl-β-D-xylopyranoside, an acceptor for GAG chain initiation and elongation. [35S]Sulfate incorporation into total medium proteoglycans was then measured. Cycloheximide completely abolished the stimulatory effect of PDGF, but if the acceptor for GAG chain synthesis was provided, the PDGF effect was fully restored (Fig. 8). These findings indicate that the enhanced GAG synthesis and chain elongation that result from PKC activation are independent from de novo protein synthesis and may involve activation of the GAG elongation enzyme pool exclusively.

FIGURE 8. Inhibition of protein synthesis does not affect PDGF stimulation of GAG synthesis in the presence of xyloside. [35S]Sulfate incorporation into CPC precipitable material following PDGF treatment in the presence of xyloside and/or cycloheximide. Cycloheximide completely blocked the PDGF effect on sulfate incorporation, but added xyloside in the presence of cycloheximide restored the increase in sulfate incorporation in the presence of PDGF. These experiments indicate that enhanced chain elongation that results from PKC activation does not involve de novo protein synthesis but rather involves activation of the GAG synthetic machinery. The data show the means ± S.D. from three experiments.

second and third lanes) reduced the electrophoretic mobility of the lower molecular weight bands previously identified to be decorin and biglycan, indicating an increase in the apparent size of these two proteoglycans. The effects of PDGF and TPA on the electrophoretic mobility of these two populations of proteoglycans were prevented by treatment of the ASMCs with H7 and unaffected by HA1004 (Fig. 7).

PKC Activation of GAG Chain Elongation Is Independent of de Novo Protein Synthesis—Previous experiments suggest that PDGF-dependent stimulation of GAG chain synthesis of not only versican but also decorin and biglycan (13, 14) is regulated independently from core protein synthesis. Therefore, we examined PDGF-stimulated GAG synthesis after inhibition of protein synthesis and/or after uncoupling core protein synthesis from GAG chain elongation. Thus, growth-arrested ASMCs were treated with various combinations of PDGF; cycloheximide, a protein synthesis inhibitor; and methyl-β-D-xylopyranoside, an acceptor for GAG chain initiation and elongation. [35S]Sulfate incorporation into total medium proteoglycans was then measured. Cycloheximide completely abolished the stimulatory effect of PDGF, but if the acceptor for GAG chain synthesis was provided, the PDGF effect was fully restored (Fig. 8). These findings indicate that the enhanced GAG synthesis and chain elongation that result from PKC activation are independent from de novo protein synthesis and may involve activation of the GAG elongation enzyme pool exclusively.

DISCUSSION

In this report, we have characterized two PDGF signaling pathways in ASMCs regulating versican core protein synthesis and post-translational modifications of the CS chains attached to versican. Furthermore, the capacity of PDGF to increase the molecular size of the three major secreted proteoglycans, versican, biglycan, and decorin by elongating GAG chains has been confirmed along with specific changes in the synthesis of chondroitin-6-sulfate to chondroitin-4-sulfate (13–16). Because the AB isoform interacts with both the α and β receptors for PDGF, we determined whether both receptors were involved in the versican response to PDGF and, if so, whether there were differences between effects on core protein synthesis and GAG chain elongation. The results clearly show that the AA and BB isoforms both caused increased mRNA transcripts for versican as well as chain elongation with the AA isoform, exhibiting less of an effect than both the BB and AB isoforms. It is well established that there are severalfold more β receptors than α receptors on ASMCs (38), so these findings are not surprising. However, these findings do indicate that the different isoforms do not control different aspects of versican biosynthesis. We now show that GAG chain elongation stimulated by PDGF is mediated by activation of PKC and that activation of PKC alone is sufficient to cause GAG chain elongation in these cells. However, activation of PKC alone is not sufficient to induce the changes in the chondroitin-6-sulfate to chondroitin-4-sulfate ratio, suggesting that other pathways control these specific post-translational modifications. PDGF-activated versican core protein expression is mediated via PKC but also via an ERK-dependent pathway. ERK activation has been associated with an ASMC proliferative and migratory phenotype (41–43). This study provides evidence that PKC and ERK are independent signaling pathways for the following reasons. First, the effect of PDGF (which activates PKC and ERK) is greater than PKC activation via the phorbol ester TPA. Second, the stimulatory effect of TPA on GAG elongation is not blocked by an ERK inhibitor. Finally, the MKK inhibitor (PD908059) is only a partial blocker of the action of PDGF to increase versican expression.

PDGF stimulates PKC activity in ASMCs, and the response is synergistic with the response to high glucose (44, 45). Of the multiple PKC isoforms present in ASMCs, pharmacological studies with the inhibitor rottlerin have shown that the PKC δ isoform is specifically associated with PDGF-mediated phosphorylation of the MAP kinase p38 (44). The phorbol ester PMA also stimulates phosphorylation and activation of p38 MAP kinase in ASMCs (45). We have recently demonstrated that transforming growth factor β-1-mediated GAG chain elongation is mediated via the canonical Smad pathway but also invokes p38 MAP kinase signaling (46). PDGF-mediated p38 MAP kinase phosphorylation in ASMCs is not blocked by the upstream ERK inhibitor PD98095 as used in the present studies (44). Thus, we can speculate that the PDGF effect on GAG elongation is mediated via activation of PKC δ and subsequent phosphorylation and activation of p38 MAP kinase. Notably, PDGF does stimulate ERK activity, but this pathway does not appear to be involved in GAG elongation.
PDGF and Proteoglycan Synthesis

Expression of versican mRNA is not increased by activation of PKC alone with phorbol ester but is almost completely abolished by inhibition of either PKC or ERK. These data imply that both PKC and ERK are required for stimulation of the expression of versican core protein. On the other hand, abolishing protein synthesis does not compromise the ability of PDGF to stimulate GAG chain synthesis if the cells are supplied with an artificial acceptor for GAG synthesis. This demonstrates that distinct signaling pathways regulate the synthesis of different aspects of individual structural components of ASMC proteoglycans. Such findings indicate the potential for developing specific targets to interfere with specific biosynthetic pathways of those macromolecules.

PDGF activation has been intensively studied in fetal lung fibroblasts, and the pathways are distinct from those identified earlier and extended here in ASMCs (47, 48). In fetal lung fibroblasts, PDGF is not mitogenic and does not stimulate the expression of proteoglycan core proteins, specifically biglycan (48). Furthermore, in fetal lung fibroblasts, GAG synthesis is dependent upon PDGF receptors, autotransphosphorylation of the receptor, and downstream phosphatidylinositol 3-kinase activation, but it is not dependent upon ERK or PKC (47). The critical fact is that mitosis of fetal lung fibroblasts is not stimulated by PDGF, whereas mature ASMCs have a well-characterized growth response to PDGF. The absence of a proliferative response to PDGF by the fetal cells suggests that signaling pathways in fetal fibroblasts with regards to a proteoglycan response may be quite different from that observed in ASMCs.

The mechanism(s) responsible for regulating GAG synthesis on proteoglycans is an important biological response, but the mechanism is presently unknown despite considerable experimental effort (3–5). CS is synthesized by the initial addition of a Xyl residue to a specific serine in the core protein followed by the sequential addition of two Gal and one GlcA residues to form the tetrasaccharide linkage region (GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-O-Ser). The sequential alternate transfer of GlcA and GalNAc forms the disaccharide repeat units of the polymerizing GAG chain. The GAG chain is subject to sulfation and epimerization to yield further structural heterogeneity. Whereas the core protein has a defined molecular weight, the GAG chains have a range of sizes conferring a range of molecular weights to the whole proteoglycan. Approximately 15 enzymes have been cloned that are involved in GAG linkage to core protein and chain polymerization. Five of these enzymes, including one identified very recently, are associated with elongation of the GAG chain (49, 50). Each of the first four elongation enzymes identified does not demonstrate polymerization activity sufficient to generate a mature GAG chain (50). A protein that has a high degree of homology with the first four chondroitin polymerization enzymes has been identified that does not have appreciable GAG polymerizing activity per se but when coexpressed with chondroitin synthetase yields appreciable in vitro polymerization activity (50). Further work has shown that chondroitin synthetase family members, when expressed in combination, exhibit distinct but overlapping substrate specificities yielding diverse outcomes for GAG synthesis (51). Thus, it will be important to further define whether PDGF affects any of these specific enzymes that modify GAG chain length. These data also address the mechanism of endogenous GAG synthesis upon which is superimposed the agonist-mediated GAG elongation that increases binding to low density lipoprotein and represents the potential underlying cause of atherosclerosis (9, 11). Recent studies by our group (52) have shown that imatinib, a PDGF receptor inhibitor, blocked GAG size increases induced by PDGF in ASMCs and reduced low density lipoprotein binding to proteoglycans both in vitro and in vivo. Sorting out the signaling pathways that control GAG chain elongation will be critical to developing a strategy to prevent proteoglycans from becoming pro-atherogenic.

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