Proteoglycan Production by Vascular Smooth Muscle Cells
From Resistance Arteries of Hypertensive Rats

Claudia Magdalena Castro, Montserrat Cecilia Cruzado, Roberto Miguel Miatello, Norma Raquel Risler

Abstract—Extracellular matrix (ECM) modifications in the vascular wall contribute to the narrowing of arteries in hypertension. Because direct evidence for the role of proteoglycans (PGs) in the pathological process of resistance-sized arteries has not already been demonstrated, we examined the effect of growth factors on secreted and membrane-bound PG synthesis by cultured mesenteric vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR) and Wistar rats. After 48 hours of stimulation with angiotensin II (Ang II), platelet-derived growth factor (PDGF-BB), and 10% fetal calf serum (FCS) or 0.1% FCS as control, PG synthesis (in dpm/ng DNA) was evaluated in the medium (M-ECM) and in the cell layer (P-ECM) by a double-isotopic label method with both [3H]-glucosamine and [35S]-sodium sulfate, which are incorporated into all complex carbohydrates or only into sulfated disaccharides, respectively. VSMC from SHR displayed a significantly lower level of synthesis of M-ECM [3H]-PGs than those of Wistar rats in all the experimental groups, including the control group (0.1% FCS), but no differences in M-ECM [35S] uptake were found in any case. In the P-ECM, Ang II was the only factor that produced a lesser effect on [3H]-glucosamine and a greater effect on [35S]-sodium sulfate uptakes in VSMC from SHR than from Wistar rats. The most prominent change seen in VSMC from SHR was an increased sulfation, assessed by [35S]/[3H] ratio, in nonstimulated cells and in response to 10% FCS and Ang II but not to PDGF-BB compared with VSMC from Wistar rats. These data indicate the existence of changes in PG modulation in the resistance vessels of SHR, which suggests that PGs may contribute to the development of structural and functional modifications in hypertensive states. (Hypertension. 1999;34[part 2]:893-896.)

Key Words: muscle, smooth, vascular ■ proteoglycans ■ growth substances ■ hypertension, essential ■ arteries

Structural changes to small and large arteries in chronic sustained hypertension include smooth muscle proliferation and extracellular matrix (ECM) modifications. Alterations to the ECM of the vascular wall are known to contribute to the narrowing of arteries and to lead to the complications of hypertension.1

Blood vessel wall ECM contains elastin, collagen, and proteoglycans (PGs);2 all of which can affect vascular resistance and, hence, blood pressure by virtue of their biomechanical properties. PGs are important nonfibrous matrix components of the arterial wall that carry unusual carbohydrates, the glycosaminoglycans (GAGs), which are composed of repeated disaccharide units and exist in different forms. Some are protein bound (the natural form); these contain sulfate associated to the carbohydrate. The other form is hyaluronic acid, which is made as a free GAG and lacks sulfate. GAGs, the sulfated ones in particular, have a strong negative charge. This charge makes it possible for GAGs to bind many substances, including growth factors. Further interesting features of this system are that the synthesis of some PGs by various kinds of cells, such as vascular smooth muscle3 or glomerular mesangial,4 is stimulated by growth factors; that many of the PGs function as modulators of growth factors;5 and that some PG–growth factor interactions are mediated by the GAG components of the PGs.6 Thus, ECM serves as a reservoir for growth and differentiation modulators. Although PGs are likely to play an important role in coordinating and regulating vessel behavior, presumably via interactions of their GAG chains or core proteins with other matrix molecules or the smooth muscle cell surface, direct evidence for their role in the pathological process of resistance-sized arteries needs to be investigated. In this study, we investigated the effect of growth factors on the synthesis of secreted and membrane-bound PG by cultured vascular smooth muscle cells (VSMC) of resistance arteries from spontaneously hypertensive rats (SHR) and Wistar rats.

Methods
Adult male (12- to 14-week-old) SHR (n=13) and Wistar rats (n=10) were used for this study. SHR were purchased at the Veterinary School of La Plata University, Argentina. All procedures were performed in accordance with institutional guidelines for animal experimentation (Animal Experimentation Committee, School of Medicine, Universidad Nacional de Cuyo). Systolic blood pressure was monitored indirectly in prewarmed slightly restrained rats by the tail-cuff method and recorded on a Grass model 7 polygraph. SHR systolic pressure (183.0±2.7 mm Hg) was signifi-
Cell Culture
Unless otherwise noted, all reagents were obtained from Sigma Chemical Co. The animals were killed by decapitation under ether anesthesia, and the mesenteric SMC were isolated according to a technique previously described. Briefly, entire mesenteric arcades, including the superior mesenteric arteries, were aseptically excised and placed in chilled HBSS with antibiotic mixture for further dissection. Five to 6 mesenteric arcades cleaned of connective tissue and fat were minced and digested in a 37°C shaker bath with the following enzyme mixture: 2 mg/mL collagenase (Worthington Biochemical Corp), 0.15 mg/mL elastase, 2 mg/mL BSA, and 0.35 mg/mL soybean trypsin inhibitor in Ham’s F-12 medium. After 2 hours, a cell suspension was obtained. Cells were grown in 10% fetal calf serum (FCS) (Gen S.A) and MEM/F-12 incubated at 37°C under humid 5% CO2/air conditions. Mesenteric SMC between the third and fifth passages were used for these experiments.

Characterization of Cultured VSMC
Although cultured VSMC exhibited the characteristic hill-and-valley growth pattern on reaching confluence, cultures were identified by the presence of positive staining with anti-smooth muscle α-actin. Complete removal of endothelial cells from mesenteric arteries was assessed by a negative staining with anti–factor VIII antibodies.

PG Evaluation
PGs were extracted and measured with techniques already described in the cultured VSMC medium and cell layer. A double-isotope method with both [35S]-sodium sulfate and [3H]-glucosamine (New England Nuclear) was used. The specific radioactivity of [3H]-glucosamine is incorporated into all complex carbohydrates, and the second radioisotope, [35S]-sodium sulfate, labels only sulfated disaccharides.

ECM Synthesis
Mesenteric VSMC were plated on 12-well plates (4×10^4 cells per well) and cultured for 3 days in MEM/F-12 supplemented with 10% FCS at 37°C under humid 5% CO2/air conditions until they reached subconfluence. VSMC were serum-starved for 48 hours in 0.1% FCS-MEM/F-12 before stimulation. Quiescent cells were then incubated with the following test materials: 100 mM L-arginine, 10 ng/mL platelet-derived growth factor (PDGF-BB), and 10% FCS or, for the control group, 0.1% FCS. Data are mean±SEM; n=6 in all groups. *P<0.05 vs 0.1% FCS group. **P<0.01 vs 0.1% FCS group. ••P<0.01 vs Wistar rat cells.

Isolation of PGs
Medium ECM: PG Secreted Into the Medium
The medium was removed from each plate, and the total volume was applied to a disposable Sephadex G-50 gel filtration column equilibrated and run in 4 mol/L guanidine hydrochloride, 0.05 mol/L sodium acetate (pH 6.0), and 2% ( wt/vol) Triton X-100. The corresponding eluted fraction was collected and analyzed for radioactivity.

Pericellular Matrix: Total Cell-Associated PG
The cell layer was rinsed in HBSS and extracted in 1 mL of 4 mol/L guanidine hydrochloride, 0.05 mol/L sodium acetate (pH 6.0), and 2% (wt/vol) Triton X-100. Chromatography was used on the solution containing the detached cells with Sephadex G-50 columns as described above. In every experiment, DNA synthesis for each treatment was determined by the Hoechst method.

The radioactivities of each medium or cell extract were counted simultaneously for [35S] and [3H] with a 3-over-2 dual-label counting method in a liquid scintillation counter (BetaRack LKB).

![Figure 1. Effect of growth factors on the synthesis of PGs secreted into the medium (M-ECM). Uptake of [3H]-glucosamine (top) and [35S]-sodium sulfate (bottom) by cultured VSMC from SHR and Wistar rats incubated with 100 nmol/L Ang II, 10 ng/mL PDGF-BB, and 10% FCS. Control group was incubated with 0.1% FCS. Data are mean±SEM; n=6 in all groups. *P<0.05 and **P<0.01 vs 0.1% FCS group. ••P<0.01 vs Wistar rat cells.](http://hyper.ahajournals.org/issue/34/5/894/fig-1)

Statistical and Data Analysis
Data are presented as total well dpm/ng DNA, mean±SEM. The statistical significance was assessed with 1- or 2-way ANOVA and a Bonferroni posttest. A P value of <0.05 was considered significant.

Results
The effect of the stimulation with different growth factors on secreted and membrane-bound total and sulfated PGs production in cultured VSMC from SHR and Wistar rats was examined. Incubation with 0.1% FCS was used as basal control in each group. Figure 1 shows the results when PGs were measured in the medium (medium ECM; M-ECM). In VSMC from both groups of rats, extracellular [3H]-PG increased significantly in the presence of 10% FCS and PDGF-BB but not in the presence of Ang II. PDGF-BB produced a greater effect than 10% FCS (P<0.01). VSMC from SHR displayed a significant lesser synthesis of [3H]-PGs than those from Wistar rats in all the treatment groups including the control group (0.1% FCS). When [35S]-PG synthesis was evaluated, PDGF-BB was the only factor that caused a significant increase in both types of VSMC compared with the 0.1% FCS control group, but no difference in [35S] incorporation between SHR and Wistar cells was found in any case.

The results obtained when total cell-associated PGs (pericellular matrix; P-ECM) were measured are shown in Figure 2. In both VSMC groups, a significant increase in [3H]-PG synthesis compared with the control treatment was produced...
only by PDGF-BB \( (P<0.01) \). The same pattern was observed for \(^{35}\text{S}\) uptake in VSMC from Wistar rats but not in those from SHR.

When SHR and Wistar rats cells were compared, no differences existed except with Ang II, which produced a lesser effect on \(^{1}\text{H}\)-PG synthesis \( (P<0.01) \) and a greater effect on \(^{35}\text{S}\) incorporation in VSMC from SHR \( (P<0.05) \).

The sulfation of GAG chains was assessed by the sulfated/total PG relationship calculated by the \([^{35}\text{S}]\)-sulfate/\([^{3}\text{H}]\)-glucosamine ratio expressed in percent \( ([^{35}\text{S}]\text{dpm/ng DNA})/([^{3}\text{H}]\text{dpm/ng DNA}) \times 100 \) (Figure 3). In VSMC from Wistar rats, only 10% FCS produced a decrease in sulfation of the medium PGs compared with the basal control group (0.1% FCS); meanwhile, in the SHR cells, both secreted and membrane-bound sulfated PGs were significantly reduced by PDGF-BB and 10% FCS. For M-ECM, this ratio was significantly greater in VSMC from SHR than from Wistar rats either in the control group (0.1% FCS) \( (P<0.01) \) or stimulated with either 10% FCS or Ang II \( (P<0.001) \) but not with PDGF-BB. The same pattern was observed in the case of P-ECM, in which 10% FCS, Ang II, and 0.1% FCS groups produced a greater \([^{35}\text{S}]\)-sulfate/\([^{1}\text{H}]\)-glucosamine ratio in SHR \( (P<0.01) \) than in Wistar rat cells.

**Discussion**

A previous study has hypothesized that ECM PGs may influence the growth and differentiation of VSMC proliferation\(^{11}\) and, consequently, may be involved in the pathophysiology of various diseases. Different ECM components have been investigated as important factors in the production of vascular proliferative disorders such as atherosclerosis and restenosis.\(^{12}\) In relation to hypertensive states, McGuffee and Little,\(^{13}\) using morphometric techniques, quantified the cell and matrix composition of normotensive rat and SHR mesenteric arteries and concluded that both cellular and matrix “remodeling” in diseased vessels is a consequence of continuous, long-term elevated blood pressure. When examining sulfated PG synthesis in several arteries from SHR and Wistar-Kyoto rats, Walker-Caproglio et al\(^{14}\) found hypertensive changes in the carotid artery but not in the superior mesenteric artery. In this study, we examine PG production by VSMC cultured from mesenteric resistance vessels.

The role that PGs play in resistance-sized arteries is still speculative. In support of the hypothesis that some PGs contribute significantly to the functional behavior of resistance arteries, the removal of partial GAGs has been reported to induce significant alterations in myogenic behavior associated with changes in passive mechanics.\(^{15}\)

The present study was designed to examine the effect of growth factors on both secreted and membrane-bound total and sulfated PG synthesis in cultured arterial smooth muscle cells obtained from the mesenteric vascular bed of SHR and Wistar rats. We found different profiles of PGs synthesis induced by various growth factors in VSMC from SHR or...
normotensive rats. The Ang II concentration (100 nmol/L) we used was selected on the basis of data reported from other authors. At this concentration, Ang II was the only growth factor that produced a greater [35S]/[3H] ratio in the presence of a complete growth factor, such as 10% FCS in VSMC of both groups and PDGF-BB in the case of SHR cells. The same variation was observed in the F-ECM PG synthesis in the SHR cells but not in those from Wistar rats. This relation between PG synthesis increase and undersulfation already has been observed in other tissues, such as human fetal mesangial and immature rat testis cells. The most prominent change seen in VSMC from SHR was an increased sulfation ratio in nonstimulated cells in response to 10% FCS and Ang II but not PDGF-BB compared with Wistar rat cells.

Our results also support the idea that cell growth and the synthesis of PGs do not always coincide. That 10% FCS is a growth-promoting agent that has a stimulatory effect on proliferation compared with 0.1% FCS is clear under the same experimental conditions. In this study, we evaluated PG production. We expressed our results as incorporated dpn/mg DNA, which excludes the differences in the growth-promoting effects of the various agents we have used. Emoto et al. studied the effect of several growth factors on pericellular PG of A10 cells and showed that smooth muscle cells in culture can proliferate without increasing PG synthesis and that PGs may enlarge without the cell growth, which suggests that cell growth and the synthesis of pericellular PG are mutually independent. Wight et al. studied the relationships that exist between proliferative states and PG synthesis, concluded that elevated PG accumulation is not only associated with growth stimulation but also with growth inhibition. Independent of the source from which the cells were obtained (SHR or normotensive rats), the effect of various growth factors on both medium and membrane-bound PG content was not synchronous with the proliferative state.

In summary, our results indicate that changes occur in PG modulation in the resistance vessels of SHR, which suggests that PGs could contribute to increased peripheral vascular resistance in hypertensive states.

Acknowledgments
This article was supported by grants from Secretaría de Ciencia y Tecnología (SECYT), Universidad Nacional de Cuyo, Mendoza, Argentina, and ANPCyT, PICT79 05-00000-01438. We gratefully acknowledge the technical assistance of Walter Manucha and the valuable assistance of Dr Alberto Binia in discussing and revising the manuscript.

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*Hypertension*. 1999;34:893-896
doi: 10.1161/01.HYP.34.4.893

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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