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Regulation of angiotensin AT_1 receptor gene expression during cell growth of vascular smooth muscle cells

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Abstract

Cell proliferation influences the expression of numerous tissue-specific genes. The angiotensin AT_1 receptor is highly expressed on vascular smooth muscle cells where it mediates cell contraction upon activation with angiotensin II. Since vascular smooth muscle cell de-differentiation leads to differential expression of several genes, we investigated the effects of cell growth on angiotensin AT_1 receptor gene expression in vascular smooth muscle cells in culture. Northern hybridization analysis revealed a decrease of angiotensin AT_1 receptor mRNA levels to approximately 20% in proliferating cells in comparison to growth-arrested cells. There is a correlative loss of membrane-associated angiotensin AT_1 receptor protein in growing cells versus non-growing cells, as assessed by saturation radioligand binding assays. In addition, the BB-isoform of platelet-derived growth factor (PDGF-BB), which induces proliferation of quiescent vascular smooth muscle cells, causes a marked down-regulation of angiotensin AT_1 receptor mRNA. These data suggest that proliferation of vascular smooth muscle cells leads to reduced angiotensin AT_1 receptor gene expression. The mechanisms underlying this process and its physiological implications remain to be defined.

Keywords: Angiotensin AT_1 receptor; Smooth muscle cell, vascular; Cell growth; Arteriosclerosis

1. Introduction

The abnormal growth of vascular smooth muscle cells is an important feature of arteriosclerosis and hypertension (Chamley-Campbell et al., 1979; Schwartz et al., 1986). Vascular smooth muscle cell de-differentiation which leads from a contractile to a proliferative cell-type is thought to be a key event in the pathogenesis of chronic vascular diseases and has therefore been subject of intense investigation. Several genes which are associated with the contractile stage of vascular smooth muscle cells such as α-actin, myosin light chain or smooth muscle-specific myosin heavy chain appear to be growth-regulated (Blank et al., 1988; Rovner et al., 1986; Monical et al., 1988). The expression of these smooth muscle cell-specific contractile proteins is in vitro inversely correlated with vascular smooth muscle cell proliferation.

The angiotensin AT_1 receptor is a G-protein-coupled receptor expressed in various tissues. The principal physiological role of angiotensin II is to enhance smooth muscle contraction and body fluid retention through activation of these angiotensin AT_1 receptors (Caponi et al., 1981; Peach, 1977; Bottari et al., 1993). In addition to this physiological role angiotensin II along with the angiotensin AT_1 receptor have been implicated in chronic vascular disease such as hypertension and arteriosclerosis which may be due to reported growth promoting effects of angiotensin II on vascular smooth muscle cells in vivo and in vitro (Berk et al., 1989; Daemen et al., 1991). Thus, there is a body of evidence that angiotensin II may influence vascular smooth muscle cell growth via angiotensin AT_1 receptor activation but only little is known about the effect of cell proliferation itself on angiotensin AT_1 receptor gene expression. Although it was recently shown that potent vascular smooth muscle cells mitogens such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and the BB-isoform of platelet-derived growth factor (PDGF-BB) cause a marked down-regulation of angiotensin AT_1 receptor gene expression in cultured vascular smooth muscle cells (Nickenig and Murphy, 1994), it is to date unclear whether cell proliferation influences angiotensin AT_1 receptor expression. Similiar to α-actin and the various myosin chain isoforms (Blank et al., 1988; Rovner et al., 1986; Monical et al., 1988), the

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angiotensin AT_1 receptor is associated with vascular smooth muscle cell contraction since angiotensin II activation of angiotensin AT_1 receptors initiates the cascade of events which ultimately leads to vascular smooth muscle cells contraction (Caponi et al., 1981; Peach, 1977; Bottari et al., 1993). In order to further elucidate the mechanisms involved in vascular smooth muscle cell de-differentiation we investigated angiotensin AT_1 receptor gene expression during cell growth.

We found out that angiotensin AT_1 receptor gene expression is substantially enhanced in quiescent versus proliferating cells. In addition, the BB-isoform of the potent vascular smooth muscle cell mitogen PDGF-BB leads to a marked decrease of angiotensin AT_1 receptor gene expression in post-confluent vascular smooth muscle cells. Together these data suggest, that vascular smooth muscle cell growth and de-differentiation leads to down-regulation of angiotensin AT_1 receptor gene expression.

2. Materials and methods

2.1. Materials

Angiotensin peptides, salts and other chemicals such as sodium chloride, sodium citrate, sodium hydroxide, and EDTA were purchased from Sigma Chemical (Deisenhofen, Germany). [methyl-^{3}H]Thymidine, [^{32}P]dCTP, Hybond N-nylon membranes and [^{125}I][Sar^1,Ile^8]angiotensin II were obtained from Amersham (Braunschweig, Germany). Antibiotics, serum and cell culture medium were purchased from Gibco BRL (Eggenstein, Germany). TRI-reagent was from Molecular Research Center (Cincinnati, OH, USA) and Dup753 was a gift from DuPont-Merck. Oligonucleotides were synthesized using Pharmacia Chemicals, with an automated DNA synthesizer (Pharmacia LKB, gene assembler plus).

2.2. Methods

2.2.1. Cell culture

Vascular smooth muscle cells were isolated from rat thoracic aorta (strain, female Wistar Kyoto, 6–10 weeks old, Charles River Wega, Sulzfeld, Germany) by the media explant method and cultured over several passages according to Ross (Ross, 1971). Cells were grown in a 5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml of penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids (100 ×) and 20% fetal bovine serum. Experiments were performed with cells from passage 5–15.

2.2.2. Measurement of DNA and protein synthesis

Vascular smooth muscle cells were seeded in 10 cm culture plates and grown to confluency. Cells were serum-deprived for 24 h and subsequently 3 μCi/ml [^{3}H]thymidine were added. Experiments were terminated 4 h after the addition of [^{3}H]thymidine by aspirating the medium and subjecting the cultures to sequential washes with phosphate-buffered saline (PBS) containing 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 10% tri-chloroacetic acid and ethanol-ether (2 : 1, v/v).

Acid insoluble [^{3}H]thymidine was extracted by adding 2 ml/dish 0.5 mol/l NaOH. 0.1 ml of this solution was mixed with 5 ml scintillator and quantified (Beckman LS 3801, Düsseldorf, Germany). Aliquots of 50 μl were used for protein determinations using the Bio-Rad (Munich, Germany) protein assay according to the method of Bradford.

2.2.3. Measurement of cell count

The experiments were terminated at various time points by washing with PBS and trypsinisation. Following two washes the cells were resuspended in 100 μl PBS. 20 μl of this cell suspension were diluted in 80 μl of PBS and stained with 20 μl Trypan-blue. The intact cells were counted in a Neubauer-cell-box using light microscopy.

2.2.4. mRNA isolation and Northern analysis

After the indicated treatments, culture medium was aspirated and the cells were lysed with 1 ml of TRI-reagent, scraped and processed according to the manufacturer's protocol in order to obtain total cellular RNA. This was quantified spectrophotometrically by measuring absorbance values at 260 and 280 nm. Ten-microgram aliquots were electrophoresed through 1.2% agarose-0.67% formaldehyde gels and stained with ethidium bromide to verify the quantity and quality of the RNA. After capillary transfer on Hybond N membranes in 3 M sodium chloride, 300 mM sodium citrate (20 × SSC) the RNA was cross-linked to the membranes using a Stratalinker 1800 (Stratagene, Heidelberg, Germany). The ethidium bromide staining patterns on these blots were inspected to ensure uniform RNA transfer. Northern blots were prehybridized for 2 h at 42°C in a buffer containig 50% deionized formamide, 0.5% sodium dodecyl sulfate (SDS), 6 × SSC, 10 μg/ml denatured salmon sperm DNA (Sigma Chemicals, Deisenhofen, Germany) and 5 × Denhardt's solution and were then hybridized for 15 h at 42°C with a random-primed, [^{32}P]dCTP-labeled, rat angiotensin AT_1 receptor cDNA probe, in the same buffer but without Denhardt's solution. The rat angiotensin AT_1 receptor cDNA template used in the random-primed synthesis of the cDNA probe was a 824-base pair fragment generated from an angiotensin AT_1 receptor cDNA (Murphy et al., 1991) by the polymerase chain reaction using the primer pair 5′GTCATGATCCCTACCCTCTACAGC-3′ and 5′-CCGTAGAACAGAGGGTTCAGGCAG-3′ and Taq polymerase. The hybridized filters were washed for 15 min at room temperature with 2 × SSC and for 15 min at 50°C with 2 × SSC-0.1% SDS and were then exposed for 5–15 h to Hyperfilm (Amersham) at −80°C.

2.2.5. Radioligand binding assays

At the beginning of each experiment culture medium was aspirated and cells were washed 3 times with PBS and collected by scraping with a rubber policeman. After centrifugation at 4°C, the cell pellets were resuspended in 1 ml ice-cold 50 mM Tris-HCl, pH 8.0, and homogenized by repeated trituration through a 22 g needle. The membranes were centrifuged and the cells were again washed, homogenized and pelleted. For binding assays 2 μg membrane protein were resuspended in a buffer containing 25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 0.1% bovine serum albumin (BSA) in a final volume of 250 μl. Total and non-specific binding points were measured in triplicates or duplicates, respectively. Non-specific binding was determined in the presence of 10 μM Dup 753. The samples were incubated for 60 min at 22°C, followed by rapid vacuum aspiration over 0.25% polyethyleneimine-treated Whatman type-GF/B filter strips and three washes with ice-cold 50 mM Tris-HCl, pH 8.0. Samples were counted in a Beckmann gamma-counter.

2.2.6. Statistical analysis

Data are presented as means ± standard error of mean (S.E.M.). Statistical analysis was performed by using the Mann-Whitney U-test.

3. Results

To avoid effects caused by differences in cell density upon initial seeding, the following measures were taken to obviate artifacts that could influence our findings: a 75 cm^2 culture flask was trypsinized 2 days after reaching confluency (approximately 3×10^6 cells). Detatched cells were seeded onto six 100 mm culture dishes (day 0). Subsequently cells were grown for 24 h before experimental procedures were initiated. Under these culture conditions cells reached confluency 4 days after splitting (day 3 of the experimental time course). Fig. 1 shows the increase in cell number per culture dish after passage. Cell proliferation was evident from day 1 to day 3: cell numbers increased 4-fold from day 1 to day 2 and increased 2-fold from day 2 to day 3. Cell proliferation gradually plateaued after reaching confluency on day 4. Fig. 2 illustrates the decrease in [^{3}H]thymidine incorporation per μg cell-protein. [^{3}H]Thymidine incorporation was maximal on day 1 which correlates with the profound cell proliferation as indicated by the cell number calculations in Fig. 1. With decreased cell proliferation [^{3}H]thymidine incorporation/μg protein decreased and plateaued after the cells reached confluency. Fig. 3 shows representative autoradiographic results from Northern analysis of a rat vascular angiotensin AT_1 receptor cDNA probe to 10 μg of total cell RNA which was extracted from vascular smooth muscle cells at the indicated time points. The expression of angiotensin AT_1 receptor mRNA was significantly reduced

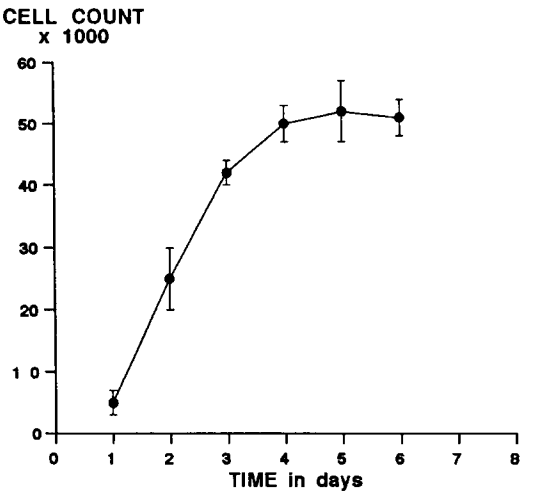

Fig. 1. Growth curve of vascular smooth muscle cells. Cells were splitted on six 100 mm culture dishes. Cells reached confluency on day 4. At the indicated time points, cells were harvested and cell count was measured using light microscopy. Each point represents mean ± S.E.M. of three seperate experiments.

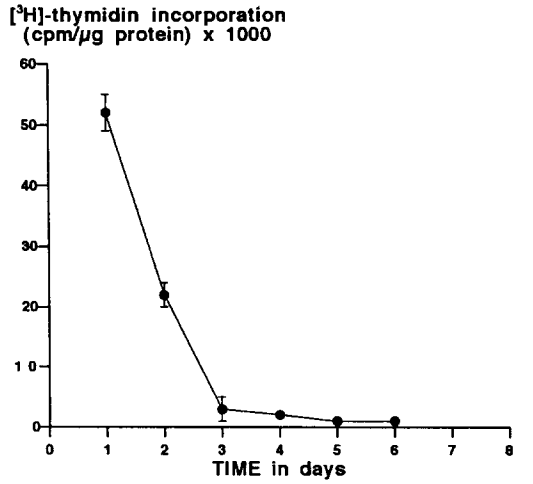

Fig. 2. DNA synthesis of vascular smooth muscle cells. Incorporation of [^{3}H]thymidine into vascular smooth muscle cells and amount of protein per culture dish was measured at the indicated time points. Each point represents mean ± S.E.M. of three seperate experiments.

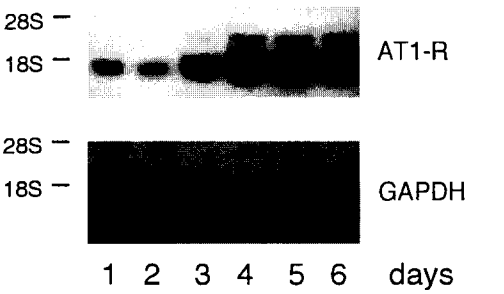

Fig. 3. Representative Northern hybridization autoradiogramms. Hybridization of an angiotensin AT_1 receptor cDNA probe to Northern blots of 10 μg total RNA extracted from vascular smooth muscle cells. Hybridization of a GAPDH cDNA probe to the same blot stripped of the angiotensin AT_1 receptor cDNA probe. Representative for three seperate experiments.

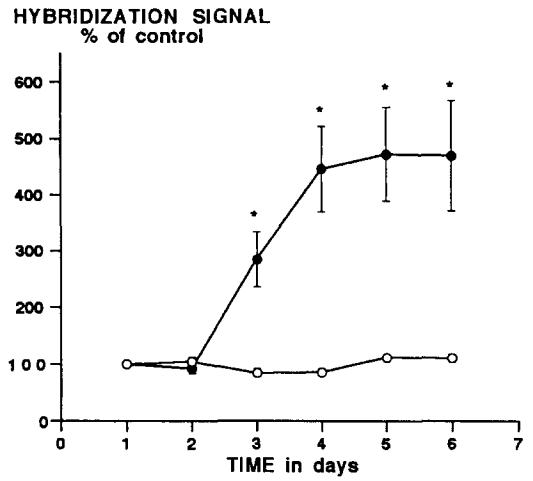

Fig. 4. Quantification of Northern hybridization signal intensity. Time course of the angiotensin AT_1 receptor mRNA levels from day 1 to day 6. Cells seeded onto 100 mm dishes were grown for the indicated times before total RNA was extracted. Northern analysis was performed as described in Materials and methods. Angiotensin AT_1 receptor mRNA hybridization signals (●) and GAPDH mRNA hybridization signals (○). Each point represents the relative hybridization signal (mean ± S.E.M.) normalized to the day 1 mRNA level of three independent experiments. $^*P < 0.05$ vs. day 1.

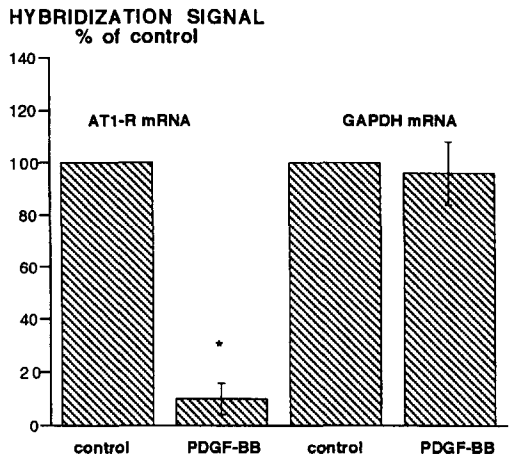

Fig. 6. Effect of PDGF-BB on angiotensin AT_1 receptor mRNA expression. Vascular smooth muscle cells grown to day 6 were incubated with either 20 ng/ml PDGF-BB or vehicle for 24 h. Total RNA was extracted and Northern analysis was performed as described in Materials and methods. The data points are expressed as a percentage of the hybridization signal from RNA from vehicle-treated cells. Each bar represents the mean ± S.E.M. of three seperate experiments. $^*P < 0.05$ vs. control.

on day 1 and day 2 and gradually increased until day 4. There was no further significant increase in angiotensin AT_1 receptor mRNA expression on day 5 and day 6. Hybridization of a glyceraldehyd-3-phosphate dehydrogenase (GAPDH) cDNA probe to the same Northern blot is also shown in Fig. 3. Compared to the angiotensin AT_1 receptor mRNA signal, GAPDH mRNA appears relatively stable over the time course of the assay. Autoradiographic data, generated from three seperate experiments, were analysed by laser densitometry. Fig. 4 illustrates graphically the level of angiotensin AT_1 receptor mRNA expression during cell growth. The angiotensin AT_1 receptor mRNA hybridization signal was minimal on day 1 (100 ± 0%) and 2 (93.5 ± 9.1%) but increased significantly until day 4 (446 ± 76.4%). Finally it plateaued on day 4 to day 6 (470 ± 98.9%) when no further cell proliferation was detectable. GAPDH mRNA showed no significant variation in this experimental set-up.

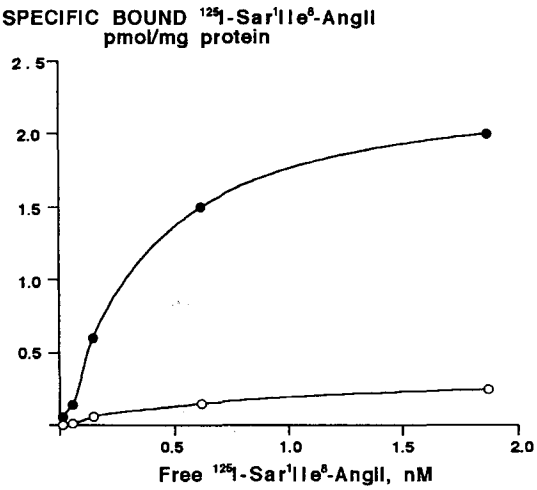

Fig. 5. Effect of cell growth on angiotensin AT_1 receptor protein. Saturation binding with [^{125}I][Sar^1,Ile^8]angiotensin II on membranes isolated from VSMC from day 1 (○) and day 6 (●). Each curve represents specific binding of the radioligand. K_d and B_{max} values reported in the text were derived from non-linear regression of the specific bound versus free data. The data are representative for three independent experiments.

Radioligand binding assays were performed in order to assess whether the decreased level of angiotensin AT_1 receptor mRNA is coincident with a loss of angiotensin AT_1 receptor protein. To test this, the total membrane receptors were measured on day 1 and day 6. Fig. 5 shows a representative saturation binding of [^{125}I][Sar^1,Ile^8] angiotensin II to membranes prepared from experimentally treated cells. Binding to membranes of cells which were grown to day 1 revealed a K_d value of 1.0 ± 0.01 nM and a B_{max} value of 0.4 ± 0.02 pmol/mg protein. Binding to membranes of cells from day 6 showed a K_d value of 1.9 ± 0.02 nM and a B_{max} value of 3.5 ± 0.7 pmol/mg protein. These binding data reveal that proliferating cells on day 1 express a significantly lower amount of angiotensin AT_1 receptor protein than quiescent cells on day 6. This finding is consistent with the observed regulatory effect of cell proliferation on angiotensin AT_1 receptor mRNA expression.

To examine whether the increase of angiotensin AT_1 receptor gene expression was caused by either decreased cell proliferation or cell-to-cell contact at confluency, we incubated confluent cells on day 6 with PDGF-BB. PDGF-BB is known to exert a strong mitogenic effect on conflu-

ent vascular smooth muscle cells thus causing a significant increase in cell proliferation and [^{3}H]thymidine incorporation (Sachinidis et al., 1990). Fig. 6 illustrates autoradiographic data from three independent experiments. Cells grown to day 6 were incubated for 24 h with PDGF-BB and subsequently angiotensin AT_1 receptor mRNA expression was measured using Northern analysis. The data reveal a marked down-regulation of angiotensin AT_1 receptor mRNA expression following the PDGF-BB treatment to $10.3 \pm 5.5\%$ in comparison to vehicle-treated cells, suggesting that the initiated cell proliferation of quiescient cells is involved in the decrease of angiotensin AT_1 receptor gene expression.

4. Discussion

In the present study we investigated the effect of vascular smooth muscle cell proliferation on angiotensin AT_1 receptor gene expression. Our findings demonstrate that in preconfluent, proliferating cells the angiotensin AT_1 receptor gene expression is markedly decreased in comparison to quiescent, confluent cells, as measured by the disapperance of mRNA transcript and cell membrane-associated angiotensin AT_1 receptors. In addition, incubation of postconfluent vascular smooth muscle cells with the mitogen PDGF-BB leads to a significant loss of angiotensin AT_1 receptor mRNA levels. These data suggest that vascular smooth muscle cell-proliferation is accompanied by decreased angiotensin AT_1 receptor gene expression.

Chronic vascular diseases are associated with abnormal growth of vascular smooth muscle cells. Proliferation of vascular smooth muscle cells leads ultimately to intimal thickening, vascular restenosis following ballon injury and participates in the development of an atherosclerotic plaque (Chamley-Campbell et al., 1979; Schwartz et al., 1986; Ross, 1993). To initiate this process, de-differentiation of vascular smooth muscle cells from a contractile to a mitotic cell-type is required. Cell contraction resembles the most important physiological feature of vascular smooth muscle cells (Chamley-Campbell et al., 1979; Creed, 1971; Chamley-Campell et al., 1981). This vascular smooth muscle cell-differentiated characteristic dissapears during cell proliferation. Although the precise mechanisms which govern vascular smooth muscle cell de-differentiation are unknown, it is well established that the expression of genes which are associated with cell contraction is down-regulated during cell growth. The expression of smooth muscle α-actin, smooth muscle myosin heavy chain isoforms, intermediate filaments, and myosin light chains appear to be inversely correlated with vascular smooth muscle cell growth (Blank et al., 1988; Rovner et al., 1986; Monical et al., 1988). The angiotensin AT_1 receptor belongs among this family of contraction-associated genes since angiotensin AT_1 receptor stimulation with angiotensin II leads to vascular smooth muscle cell contraction. As shown in this study, angiotensin AT_1 receptor gene expression is down-regulated during vascular smooth muscle cell proliferation which is similiar to the expression of smooth muscle-specific contractile proteins. We showed previously, that stimulation of vascular smooth muscle cells with potent mitogens such as PDGF-BB, EGF as well as bFGF causes down-regulation of angiotensin AT_1 receptor gene expression. This led us to the hypothesis that growth factor-induced vascular smooth muscle cell de-differentiation is required for angiotensin AT_1 receptor down-regulation. This notion is supported by the present data, since effects of the growth factors unrelated to the induction of cell growth were obviated. Nevertheless, the underlying mechanisms and intracellular events which lead to the proliferation-associated decrease of angiotensin AT_1 receptors in vascular smooth muscle cells remain unclear. Furthermore, it is at present unknown whether angiotensin AT_1 receptor gene repression is a prerequisite for vascular smooth muscle cell growth, since the possibility of simple coincidence of angiotensin AT_1 receptor down-regulation and vascular smooth muscle cell proliferation cannot be excluded.

It is important to note that our experiments were performed in cultured vascular smooth muscle cells of the rat aorta. Extrapolation to in vivo models needs to be considered with caution and requires additional confirmation. But it is still attractive to speculate that angiotensin AT_1 receptor gene expression may be depressed in replicating cells within a vascular lesion following ballon injury. Therefore it may be of interest to investigate angiotensin AT_1 receptor expression in these lesions, since decreased angiotensin AT_1 receptor levels could explain the missing success of angiotensin-converting enzyme inhibitors following ballon injury in humans (MERCATOR Study Group, 1992).

Finally, the present data have implications regarding design and interpretation of experiments examining angiotensin AT_1 receptor gene regulation. Care should be taken to control the state of confluency of vascular smooth muscle cells, since profound variations in angiotensin AT_1 receptor gene expression may be caused not only by the investigated intervention but also by the different state of cell proliferation. In conclusion, vascular smooth muscle cell proliferation causes down-regulation of angiotensin AT_1 receptor gene expression. The elucidation of the mechanisms which govern this gene regulation requires further investigation and may lead to a better understanding of the cellular events involved in smooth muscle cell growth.

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