Mechanism for Aldosterone Potentiation of Angiotensin II–Stimulated Rat Arterial Smooth Muscle Cell Proliferation

Fang Xiao, John R. Puddefoot, Stewart Barker, Gavin P. Vinson

Abstract—After earlier studies in which secretion of aldosterone was demonstrated to be important in rat arterial smooth muscle cell (RASMC) proliferation in vitro, the presence of both 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) gene transcription were shown in these cells by real-time reverse transcription–polymerase chain reaction (RT-PCR). In proliferation studies, tritiated thymidine incorporation into RASMC and RASMC cell number were both significantly increased by angiotensin II (Ang II) (10⁻⁷ mol/L) compared with controls (P<0.01), but this effect was inhibited by the 3β-hydroxysteroid-dehydrogenase inhibitor trilostane (10⁻⁶ mol/L and 10⁻⁵ mol/L, P<0.05). Aldosterone alone added to RASMC did not significantly change tritiated thymidine incorporation when compared with controls, but the Ang II–induced increase was significantly enhanced by aldosterone at 10⁻¹⁰ mol/L and 10⁻⁸ mol/L (P<0.05). Neither corticosterone nor 18-hydroxydeoxycorticosterone had any such potentiating effect. RT-PCR analysis and real-time quantitative RT-PCR revealed an increase of Ang II type-1 (AT₁) receptor mRNA in RASMC treated by aldosterone (10⁻⁸ mol/L) compared with untreated controls, and this was correlated with a small but significant increase in AT₁ receptor protein (P<0.05), as assessed by immunoblotting analysis. These data confirm that steroid production by RASMC is critical in the response to Ang II, and the data support the view that aldosterone specifically is required for the full proliferative response to Ang II in RASMC. One way it may act is by modulating the expression and functions of the AT₁ receptor. (Hypertension. 2004;44:340-345.)

Key Words: muscle, smooth, vascular ■ angiotensin II ■ mineralocorticoids ■ receptors, angiotensin ■ hypertension, essential

After recent descriptions of aldosterone formation in sites other than the adrenal, for example in the vasculature, its biosynthetic origins and the specific functions of tissue, as opposed to circulating, aldosterone becomes of interest. Although it is known that aldosterone may play a role in raising blood pressure by modulating human vascular smooth muscle tone, it is less clear whether it is also involved in vascular smooth muscle cell proliferation.

Over the past 15 to 20 years, a number of observations have suggested that the actions of angiotensin II (Ang II) extend beyond those of a transiently acting vasoconstrictor and aldosterone secretogogue. Ang II is a powerful mitogen for many cell types and a potent competence or progression factor, stimulating transition from the G₀-G₁ phase in the cell cycle, leading in certain conditions to increased DNA synthesis and mitogenesis in combination with other growth factors. Ang II also plays crucial roles in the pathogenesis of atherosclerosis and hypertension. Ang II type-1 (AT₁) receptor content of vascular smooth muscle cells is increased in atherosclerotic lesions and the neointima after balloon injury.

Recent interest in the role of aldosterone in cardiovascular function has been stimulated by the knowledge that aldosterone is associated with fibrosis, inflammation, and vascular smooth muscle cell contractility, and that the use of aldosterone antagonists as well as angiotensin-converting enzyme inhibitors or angiotensin antagonists brings additional benefit in cardiovascular disease.

Aldosterone synthase (CYP11B2) mRNA and CYP11B2 activity and 11β-hydroxylase (CYP11B1) mRNA have been reported to be expressed in human endothelial cells, rat mesenteric arteries, and heart, although the latter finding was not confirmed by Gomez-Sanchez et al. We previously reported that Ang II stimulates aldosterone synthesis in rat arterial smooth muscle cell (RASMC), and the first part of the present study was directed to examining the nature of the CYP11B species that may be involved. We also previously showed that Ang II–stimulated RASMC proliferation is blocked by the antagonist spironolactone. Although a direct effect of aldosterone on cell proliferation has not been shown, it has been reported that aldosterone enhances Ang II–induced protein synthesis in RASMC and human vascular...
smooth muscle cells. Accordingly, the present study also addresses the possibility that RASMC proliferation induced by Ang II may be similarly potentiated by aldosterone. In addition, the possibility that aldosterone may affect expression of AT1 receptor gene transcription and expression was examined.

To test whether de novo steroidogenesis is important in the actions of Ang II, this study also addresses the effects of triolostane, a 3β-hydroxysteroid-dehydrogenase inhibitor, on the proliferation of RASMC stimulated by Ang II.

**Methods**

**RASMC Preparation**

Male Wistar rats, 200 to 300 grams, were obtained from commercial suppliers. Animals were euthanized and adrenal tissue was prepared as previously described. RASMC were obtained by an explant method from the aorta using culture conditions as described previously. Cells were incubated with RPMI-1640 culture medium (Sigma) containing 100 U/mL of penicillin (Gibco, Paisley, Strathclyde, UK), 100 μg/mL streptomycin (Gibco), 4 μmol/L L-glutamine (Gibco), and 20% fetal bovine serum (Sigma). Experiments were performed after 3 to 5 passages. Adrenal tissue was also obtained from Wistar rats and separated into capsule (largely glomerulosa) and inner zone (fasciculata/reticularis/medulla) fractions as previously described.

**Real-Time Reverse Transcription–Polymerase Chain Reaction**

**RNA Extraction**

Total cellular RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol and quantified spectrophotometrically by measuring absorbency at 260 and 280 nm. The total RNA purity (A260/A280) was between 1.6 and 1.9. The quality of RNA was confirmed by ethidium bromide staining after 1% agarose gel fractionation. Extracted RNA was stored at −70°C until required.

**Primer Preparation**

For amplification of CYP11B1, primers were derived from the Genbank database. For CYP11B2, AT, receptor and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were chosen from previously published sequences (more details can be found in an online supplement available at http://www.hypertensiona.org/).

**Real-Time Reverse Transcription–Polymerase Chain Reaction Protocol**

Using Brilliant SYBR Green QRT-PCR Master Mix Kit, 1-step based on real-time detection of accumulated fluorescence (Mx300P; Stratagene, Amsterdam), Real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed according to the manufacturer’s protocols. Three micrograms of total cellular RNA was used per reaction. For CYP11B1 and CYP11B2, RT-PCR was performed with the following time courses: 50°C for 30 minutes for the first-strand cDNA synthesis, 95°C for 10 minutes, 45 cycles of 95°C for 30 seconds, 56°C (CYP11B1) or 66°C (CYP11B2) for 1 minute, and 72°C for 1 minute for amplification. The amplified products were subjected to a stepwise increase in temperature from 55°C to 95°C and dissociation curves were constructed. No template reactions were performed to screen for contamination of reagents or false amplification of primer dimers. Plasmids pSVL-11B1 and pSVL-11B2 (gift from Professor Okamoto, Osaka University Medical School, Japan) containing cDNAs encoding CYP11B1 and CYP11B2, respectively, and adrenal tissues were used as positive controls. For AT, receptor and GAPDH, RT-PCR was performed with the same time courses as CYP11B1. Target mRNAs were quantified by measuring the threshold cycle (when fluorescence is statistically significantly above background) and reading against a calibration curve. The relative amount of each mRNA was normalized to the housekeeping gene (GAPDH) mRNA. Each sample was tested in triplicate.

**RT-PCR Protocol**

RT was performed at 23°C for 20 minutes and 42°C for 60 minutes using 5 μg RNA, 1 μL reverse transcriptase (Moloney murine leukemia virus reverse-transcriptase; 200 U/μL; Gibco) and 1 μL random hexamers (3 μg/μL; Gibco) in a 20-μL reaction mixture, as described elsewhere (for more information see the online supplement).

**Cell Proliferation**

**Cell Number**

Cell count experiments were performed as described previously, with minor modification. Briefly, a suspension of RASMC (0.5×10^5 cells/mL) were prepared and treated with Ang II (10^−4 mol/L) with or without triolostane (10^−4 to 10^−2 mol/L) in 20% fetal bovine serum RPMI-1640 without further change of medium, using 3 wells per group. Experiments were terminated after an additional 48-hour incubation. Cells were counted in a hemocytometer using light microscopy.

**Tritiated Thymidine Uptake**

Thymidine incorporation experiments were performed as described previously. Briefly, the cells were synchronized in the G0/G1 phase by serum deprivation for 24 hours. The quiescent (serum-deprived) cells were incubated with serum-free medium containing hormonal stimulation, as required. The cells were collected and 3H-methylthymidine uptake was assayed as described previously.

**Immunoblotting Analysis**

Protein from cultured cells was prepared as previously described and estimated using DC Protein Assay (Bio-Rad). Protein (10 μg/lane) was then separated on 7.5% polyacrylamide gel with Precision Protein Standard (Bio-Rad) and positive controls (adrenal cortex homogenate) before transfer to Hybond-ECL nitrocellulose membrane (Amersham) for 1 hour at 100 v at 4°C. Protocols for immunoblotting were those previously described, using AT, receptor-specific antiserum (AT1: sc-579; Santa Cruz Biotechnology, Wiltshire, UK) diluted 1:750, as primary antibody, and donkey antirabbit IgG as secondary antibody. Bands were quantified by Image Acquisition and Analysis software (UVP).

**Statistical Analysis**

Values are expressed as means±SEM. The level of significance for difference between means was evaluated by ANOVA or by Student t test as appropriate.

**Results**

**CYP11B1 and CYP11B2 Gene Transcription in Cultured RASMC**

Using CYP11B1 primers, real-time RT-PCR with mRNA from the pSVL-11B1 plasmid and from adrenal fasciculata cells resulted in a major peak at melting temperature (Tm) 85°C and a minor peak at 81°C. The same 2 peaks were also amplified from RASMC mRNA (Figure 1A). In the absence of template, or in the presence of an inappropriate pSVL-11B2 template, only single fluorescence peaks corresponding to primer dimer melting at lower Tm, at ≈75°C, were seen (Figure 1A). Using CYP11B2 primers, a single fluorescence peak with the Tm 83°C appropriate for the expected product was seen in the presence of pSVL-11B2 plasmid, adrenal glomerulosa, and RASMC (Figure 1B). A peak at Tm 75°C was obtained in the absence of template or in the presence of...
the inappropriate pSVL-11B1 mRNA, representing primer dimer formation (Figure 1B).

**Cell Proliferation**

Tritiated thymidine incorporation into RASMC treated with Ang II 10^{-7} mol/L was significantly increased compared with that of control (P<0.01). This effect was inhibited by trilostane 10^{-6} (P<0.05) and 10^{-5} (P<0.01), but not at 10^{-9}, 10^{-8}, and 10^{-7} mol/L (Figure 2A).

Cell number was also significantly increased compared with that of control in groups treated with Ang II 10^{-7} mol/L (P<0.01), and this effect too was inhibited by trilostane at 10^{-6} and 10^{-5} mol/L (P<0.05), but not at 10^{-9}, 10^{-8}, and 10^{-7} mol/L (Figure 2B).

Aldosterone alone did not significantly change the tritiated thymidine incorporation when compared with controls (Figure 3), but the Ang II–induced increase in tritiated thymidine incorporation into RASMC was significantly enhanced by aldosterone 10^{-10} and 10^{-8} mol/L (P<0.05), but not at 10^{-12} and 10^{-10} mol/L. Neither corticosterone nor 18-hydroxydeoxycorticosterone at concentrations up to 10^{-6} mol/L showed any similar potentiation of the proliferative response to Ang II when used in the same way as aldosterone (data not shown).

**AT1 Receptor Expression**

Western analysis of 10 μg protein from RASMC treated with or without aldosterone (10^{-8} mol/L) in serum-free medium for 48 hours revealed staining of a band with molecular weight of 54 kDa and further bands 75 kDa (Figure 4A). Such variation in molecular weight may reflect differences in glycosylation; 54-kDa and 75-kDa bands were also present in adrenal tissue homogenates (Figure 4B), and for accurate quantification, immunoblotting of serially diluted adrenal cortex homogenates was prepared and confirmed a linearity of the 54-kDa band when the range of 0 to 10 μg protein was used in the methods. Quantification of the 54-kDa band in aldosterone-treated RASMC was significantly increased compared with that of control (P<0.05) (Figure 4C).

**Transcription of AT1 Receptor mRNA**

AT1 receptor mRNA was detected by RT-PCR (Figure 5A and 5C) and real-time quantitative RT-PCR (Figure 5D) in RASMC incubated with or without aldosterone 10^{-8} mol/L for 48 hours. To test the specificity of amplified sequences, the AT1 receptor PCR products were digested with restriction enzymes and the specific restriction products were detected, showing the size predicted for AT1 of 356 bp and 679 bp (Figure 5B). Related to GAPDH transcription, abundance of
AT$_1$ receptor mRNA was significantly greater in RASMC treated with aldosterone 10$^{-8}$ mol/L than that of control (P<0.05) (Figure 5C). The results of real-time quantitative RT-PCR were similar to those of RT-PCR (Figure 5D). The threshold cycle ranged from 16 to 17 in the assays for AT$_1$ receptor, and ranged from 14 to 15 for GAPDH.

**Discussion**

Physiologically, the main site for aldosterone synthesis is the adrenal cortex. However, increasing evidence suggests that modulation of blood pressure and cardiovascular homeostasis is effected not only via the effects of circulating components formed within the adrenal but also through local synthesis.\(^1,2,17,26\)

In the present study, clear evidence for the transcription of genes coding for both CYP11B1 and CYP11B2 was found for the first time in cultured RASMC, consistent with our previous finding of aldosterone secretion (Figure 1). Although it remains unclear how much of the steroidogenic pathway normally associated with the adrenal cortex is present, both the evidence of immunoassayable aldosterone previously obtained\(^18\) and the actions of the 3β-hydroxysteroid dehydrogenase inhibitor, trilostane, in the present study, indirectly support the view that steroid synthesis de novo can take place. Thus, we also demonstrated that the Ang II–induced increase in tritiated thymidine incorporation into RASMC was significantly inhibited by trilostane at 10$^{-6}$ and 10$^{-7}$ mol/L (Figure 2A). In addition, the significant increase in RASMC cell number at 10$^{-7}$ mol/L Ang II compared with that of control was also inhibited by trilostane at 10$^{-6}$ and 10$^{-7}$ mol/L (Figure 2B). These findings suggest that it is likely that steroid synthesized within the vascular tissue participates in regulation of RASMC proliferation in an autocrine or paracrine manner rather than via the systemic circulation, which is a conclusion consistent with those of Duprez et al\(^26\) and Berk\(^6\) in recent reviews. Our previous results have indicated not only that RASMC can produce immunoreactive aldosterone but also that this was increased by Ang II. Furthermore, Ang II–stimulated proliferation of these cells was inhibited by spironolactone, suggesting that the action of Ang II is at least partly mediated by aldosterone.\(^18\)

The results presented here showed that although aldosterone alone did not significantly change tritiated thymidine incorporation in RASMC (Figure 3), the Ang II–induced increase was significantly enhanced by aldosterone in a specific manner, because neither corticosterone nor 18-hydroxycorticosterone had similar actions. The present data were consistent with those of others\(^1,19\) who showed an enhancement by aldosterone of Ang II–induced protein synthesis in RASMC. The apparent lack of significant effect of aldosterone at 1 µmol/L, in contrast to its potentiation of the proliferative action of Ang II at lower concentrations, suggests a biphasic response.

Most Ang II effects are mediated via the AT$_1$ receptor.\(^6,7\) AT$_1$ receptor expression is regulated by Ang II itself and by heterologous regulation.\(^27\) In the present study, we explored the effects of aldosterone on the expression of AT$_1$ receptors. Our data demonstrate that aldosterone caused upregulation of

**Figure 3.** Aldosterone alone did not affect $^3$H-methylthymidine incorporation into RASMC. Aldosterone at 10$^{-12}$ and 10$^{-8}$ mol/L enhanced the incorporation induced by Ang II (10$^{-7}$ mol/L), but not at 10$^{-11}$ and 10$^{-6}$ mol/L. Values are means±SEM, N=4 per group. ANOVA P<0.001; Student t test **P<0.01, *P<0.05.

DPM indicates disintegrations per minute.

**Figure 4.** Western blot analysis of AT$_1$ receptor protein expression in cultured RASMC incubated with or without aldosterone 10$^{-8}$ mol/L for 48 hours. A, Results show staining of expected band at 54 kDa, with multiple bands visible over 75 kDa. B, Results analysis of positive control with serially diluted adrenal cortex homogenates show linearity over the range 0 to 10 µg (R=0.9798). C, The 54-kDa band was quantified. The expression of AT$_1$ receptor protein in treated RASMC was significantly increased compared with that of control (P<0.05). Data are means±SEM from 3 experiments. Values are expressed as a percentage of control culture. *P<0.05 versus control culture. A indicates aldosterone-treated cells; C, control culture.
creased transcription of mRNA coding for the AT₁ receptor in RASMC culture medium,18 corticosterone was used. It is remarkable that although aldosterone output was not or it was below detection limits.

In the rat, the finding of CYP11B1 and CYP11B2 alone is insufficient to account for aldosterone production, given that, in the adrenal at least, a minimum of 3 cytochrome P-450 species and 1 dehydrogenase/isomerase system are required for aldosterone synthesis. However, the presence of stimulatory immunoreactive aldosterone18 and the present findings of the action of trilostane suggest these enzymes must be all present. It is remarkable that although aldosterone output was detected in the RASMC culture medium, corticosterone was not or it was below detection limits.

Perspectives
Taken together, the data show that aldosterone potentiates the actions of Ang II on RASMC proliferation and maintains AT₁ receptor expression and function. These findings suggest mechanisms for the now well-recognized actions of anti-aldosterone drugs in the treatment of vascular disease.14 It will be important in future work to address not only the biosynthetic route for aldosterone in the vasculature but also its regulation to better-understand how its importance differs from that of systemic aldosterone. In addition, the mechanism by which aldosterone affects AT₁ expression requires elucidation. Finally, there are possible clinical implications for these studies in the light of recent data from the trials performed by Rales and Ephesus.31

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References


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