After recent descriptions of aldosterone formation in sites other than the adrenal, for example in the vasculature,1,2 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,3,4 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.5 Ang II is a powerful mitogen for many cell types6,7 and a potent competence or progression factor, stimulating transition from the G0-G1 phase in the cell cycle, leading to increased DNA synthesis and mitogenesis in combination with other growth factors.8 Ang II also plays a crucial role 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.9,10

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,11–13 and that the use of aldosterone antagonists as well as angiotensin-converting enzyme inhibitors or angiotensin antagonists brings additional benefit in cardiovascular disease.14

Aldosterone synthase (CYP11B2) mRNA and CYP11B2 activity and 11β-hydroxylase (CYP11B1) mRNA have been reported to be expressed in human endothelial cells,15 rat mesenteric arteries,16 and heart,2 although the latter finding was not confirmed by Gomez-Sanchez et al.17 We previously reported that Ang II stimulates aldosterone synthesis in rat arterial smooth muscle cell (RASMC),18 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.18 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 RASMC19 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 evaluated.

To test whether de novo steroidogenesis is important in the actions of Ang II, this study also addresses the effects of trilostane, 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, AT1 receptor and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were chosen from previously published sequences (more details can be found in an online supplement available at http://www.hypertensionaoh.org). For amplification of CYP11B1, primers were derived from the pSVL-11B1 plasmid and from adrenal fasciculata (largely glomerulosa, and RASMC (Figure 1B). A peak at Tm 75°C appropriate for the expected product was seen. 23 Extracted RNA was used per reaction. For CYP11B1 and CYP11B2, RT-PCR was performed with the same time courses as CYP11B1. Target mRNAs were amplified from RASMC mRNA (Figure 1A). In the absence of RT-PCR, CYP11B2 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 85°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

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).

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 AT1 receptor-specific antisera (AT1, 1:306; 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).

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 AT1 receptor-specific antisera (AT1, 1:306; 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 85°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

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).
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).

**AT\(_1\) Receptor Expression**

Western analysis of 10 \(\mu\)g 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 \(\mu\)g 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 AT\(_1\) Receptor mRNA**

AT\(_1\) 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 AT\(_1\) receptor PCR products were digested with restriction enzymes and the specific restriction products were detected, showing the size predicted for AT\(_1\) of 356 bp and 679 bp (Figure 5B). Related to GAPDH transcription, abundance of 356 bp and 679 bp AT\(_1\) receptor mRNA was significantly increased compared with that of control (\(P<0.01\)).
AT<sub>1</sub> receptor mRNA was significantly greater in RASMC treated with aldosterone 10<sup>-8</sup> 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<sub>1</sub> 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.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 obtained18 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<sup>-6</sup> and 10<sup>-7</sup> mol/L (Figure 2A). In addition, the significant increase in RASMC cell number at 10<sup>-7</sup> mol/L Ang II compared with that of control was also inhibited by trilostane at 10<sup>-6</sup> and 10<sup>-7</sup> 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<sup>26</sup> and Berk<sup>6</sup> 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-hydroxyldeoxycorticosterone had similar actions. The present data were consistent with those of others<sup>1,19</sup> 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<sub>1</sub> receptor.6,7 AT<sub>1</sub> 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<sub>1</sub> receptors. Our data demonstrate that aldosterone caused upregulation of
AT1 mRNA (Figure 5) and protein (Figure 4). Although Della Bruna et al.28 found that mineralocorticoid treatment decreased transcription of mRNA coding for the AT1 receptor in rat heart, the present results are consistent with those of Robert et al.,29 who conversely reported increased cardiac AT1 protein and gene transcription expression in aldosterone/salt-treated rats. It is possible that such differences may arise from variation in experimental protocols, and certainly it is also possible that other mechanisms occur in other tissues, for example, the adrenal, in which aldosterone had no effect on magnitude of AT1 expression, although apparently altering its location.30

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 stimulable immunoreactive aldosterone18 and the present findings performed by Rales and Ephesus.31

**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

**Acknowledgments**

We are grateful to Professor Mitsuhiro Okamoto and to Dr. Hiroshi Takemori, Department of Biochemistry and Molecular Biology, Osaka University, Osaka, Japan, for the gift of the CYP11B1 and CYP11B2 plasmids. This work was supported by Stegram Pharmaceuticals, Ltd.

**References**


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

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

*Hypertension*. 2004;44:340-345; originally published online August 9, 2004; doi: 10.1161/01.HYP.0000140771.21243.ed

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
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:
http://hyper.ahajournals.org/content/44/3/340

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2004/08/27/01.HYP.0000140771.21243.ed.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Hypertension* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Hypertension* is online at:
http://hyper.ahajournals.org//subscriptions/