Role of Prostaglandins in Angiotensin-Induced Steroidogenesis
Absence of an Effect by Prostaglandin E₂

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SUMMARY Recently, we have found that the prostaglandin synthesis inhibitor, indomethacin, reduced basal and angiotensin-stimulated aldosterone release. To further test the possibility that prostaglandins (PGs) function as mediators of adrenal steroidogenesis, we examined the release of aldosterone, PGE₂, and PGF₄α under basal and stimulated conditions in isolated adrenal capsular cells in vitro. Angiotensin II and III caused a dose-related increase in aldosterone release without significantly altering the release of PGE₂ or PGF₄α. Indomethacin inhibited basal, angiotensin II, and angiotensin III-induced steroidogenesis by 40%, 15%, and 52% respectively. Additionally, it inhibited the release of PGE₂ by 60% in the control and angiotensin-treated cells. In indomethacin-treated cells, PGE₂ stimulated aldosterone release in supraphysiologic doses; however, its steroidogenic effect was not additive with angiotensin II. The prostaglandin precursor, arachidonic acid, increased the adrenal synthesis of PGE₂ and PGF₄α in a dose-related manner without altering the synthesis of aldosterone. Similarly, the prostaglandin endoperoxide PGH₂ increased the synthesis of PGE₂ by 250-fold, yet failed to alter aldosterone synthesis. These findings indicate that PGE₂ does not mediate or modulate basal or angiotensin-stimulated steroidogenesis. Furthermore, it would appear that indomethacin may inhibit adrenal steroidogenesis via a mechanism other than inhibition of prostaglandin synthesis.

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KEY WORDS • indomethacin • arachidonic acid • prostaglandin H₂ • prostaglandin F₄α • angiotensin II • angiotensin III

RECENTLY, we have found that the prostaglandin synthesis inhibitor indomethacin inhibited the release of aldosterone by angiotensin II and III in normal and sodium-depleted conscious rats and in rat adrenal cell suspensions. Another prostaglandin synthesis inhibitor, meclofenamate, inhibited angiotensin III but not angiotensin II-induced steroidogenesis. Since both drugs simultaneously inhibited the urinary excretion of prostaglandin E₂ (PGE₂) and prostaglandin F₄α (PGF₄α) reduced the adrenal prostaglandin content and reduced the adrenal conversion of ¹⁴C-arachidonic acid to ¹⁴C-PGE₂ and ¹⁴C-PGF₄α, it was suggested that adrenal prostaglandins mediate a portion of the steroidogenic effects of the angiotensins. This contention was also suggested by the observations of Saruta and Kaplan that PGE₂ stimulated the release of aldosterone from adrenal cortical slices, an effect that was additive with angiotensin II. Subsequent studies confirmed the steroidogenic effects of PGE₂ and PGE₂ in vitro but not in vivo; however, it should be mentioned that in each of the in vitro studies supraphysiologic doses of the prostaglandins were required for the release of aldosterone.

Our present studies were designed to further test the possibility that angiotensin stimulates the adrenal production of PGE₂ and that this PGE₂ augments the steroidogenic activity of the peptide. To accomplish this, we examined the effects of angiotensin II and III on the release of aldosterone, PGE₂, and PGF₄α in adrenal cell suspensions. Also, the effects of the precursors of these prostaglandins, arachidonic acid and prostaglandin cyclic endoperoxide (PGH₂), on the release of aldosterone were determined.

Methods

Male Sprague-Dawley rats (225–300 g; Simonsen Laboratories) were used in these studies. The rats were maintained on a standard Wayne Rat Chow diet containing 142 mEq/kg of sodium and 290 mEq/kg of potassium and tap water ad libitum.
Adrenal cell suspensions were prepared by the method of Sarstedt et al.\(^1\) with several modifications.\(^1\) Rats were killed by decapitation, and their adrenal glands were removed and placed in potassium-free medium 199. The adrenals were separated from the adhering fat, and the capsules removed. The capsular tissue was minced and incubated in potassium-free medium 199 containing 0.5 mg/ml of trypsin (Sigma) for 15 minutes in a Dubnoff metabolic shaker at 37°C under an atmosphere of 95% \(O_2\) and 5% \(CO_2\). The tissue was then washed twice with potassium-free medium 199 containing 1 mg/ml of lima-bean trypsin inhibitor and 2 mg/ml of bovine serum albumin. In the studies presented here, the final incubation buffer contained 0.5 mg/ml of trypsin (Sigma), 0.05 mg/ml of ribonuclease (Sigma), and 1 mg/ml of bovine serum albumin. After 30 and 60 minutes of incubation, the cells were dispersed by repeated (30 times) pipetting. The suspension was then washed twice with potassium-free medium 199 containing 2 mg/ml of bovine serum albumin and resuspended in medium 199 containing 1 mg/ml of collagenase (Worthington), 0.05 mg/ml of deoxyribonuclease (Sigma), 0.05 mg/ml of ribonuclease (Sigma), and 2 mg/ml of bovine serum albumin, and incubated for 1 hour in a Dubnoff metabolic shaker at 37°C under 95% \(O_2\) and 5% \(CO_2\). At the end of the incubation period, the cell suspensions were centrifuged and the supernatant aspirated. The tissue was minced and incubated in potassium-free medium 199 containing 1 mg/ml of lima-bean trypsin inhibitor and 2 mg/ml of bovine serum albumin, and resuspended in medium 199 containing 4.5 mEq/liter of potassium and 2 mg/ml of bovine serum albumin. In the studies with arachidonic acid and PGH\(_4\), the bovine serum albumin was omitted from the final incubation buffer since it is known to bind fatty acids and convert PGH\(_4\) to PGD\(_2\). The adrenal cells were counted with a hemocytometer, and the number of viable cells determined by exclusion staining of trypan blue. Using this method, we obtained approximately 300,000 viable cells per adrenal capsule, and the viability of the cells was approximately 94%.

We then placed 0.5 ml containing 500,000 to 700,000 cells in a Dubnoff metabolic shaker at 37°C under an atmosphere of 95% \(O_2\) and 5% \(CO_2\). All compounds were added in a constant volume of 10 \(\mu\)l, and an equal volume of their vehicle was added to the control tubes. Following these additions, the cells were incubated for 1 hour. At the end of the incubation period, the cell suspensions were centrifuged and the supernatant assayed for aldosterone by direct radioimmunoassay. The results are expressed as nanograms (ng) of aldosterone/10\(^6\) cells/hr. The remainder of the supernatant was combined from two incubation tubes and assayed for PGE\(_2\) and PGF\(_{2\alpha}\) by radioimmunoassay.

Prostaglandins E\(_2\), F\(_{2\alpha}\) and F\(_{20}\) were measured by the method of Dray et al.\(^4\) with several modifications. The PGE\(_2\) and PGF\(_{2\alpha}\) antisera were produced in our laboratory in rabbits immunized against a prostaglandin-thyroglobulin conjugate. The conjugate was prepared by the method of Jaffe and Behrman,\(^10\) mixed with Freund's complete adjuvant (Grand Island Biologicals), and injected monthly in multiple subcutaneous sites in rabbits. The cross reactivities of the antibodies with known prostaglandins are listed in table 1.

The assay consisted of extracting 1 ml of incubation media (containing 1000 cpm of \(^3\)H-PGE\(_2\) and \(^3\)H-PGF\(_{2\alpha}\))-New England Nuclear) with 10 ml of ethyl acetate-cyclohexane (50:50) after acidification to pH 3.0 with glacial acetic acid. The organic phase was then removed and evaporated to dryness at 30°C under nitrogen. The extract was reconstituted in a benzene-ethyl acetate-methanol mixture (60:40:10) and placed on an 0.8 X 10 cm silicic acid column (Sigma, SIL-200). The prostaglandins were then eluted with solvents of increasing polarity with PGA, PGF\(_{2\alpha}\), 6-keto-PGF\(_{1\alpha}\), 15-keto-PGDF\(_{2\alpha}\), PGE\(_2\) in the first fraction, PGE\(_2\) in the second, and PGF\(_{2\alpha}\) in the third (fig. 1). These column eluates were dried at 30°C under nitrogen and reconstituted in 1.0 ml of phosphate-buffered saline containing 0.1% polyvinylpyrrolidone. Radioimmunoassay was performed by adding 0.1 ml of the unknown to 3000 cpm of \(^3\)H-PGE\(_2\) or \(^3\)H-PGF\(_{2\alpha}\) and 0.1 ml of the prostaglandin antisera (titer 1:8000 for PGE\(_2\) and 1:30,000 for PGF\(_{2\alpha}\)) and incubated overnight at 4°C. The bound and free prostaglandins were separated by the addition of dextran-coated charcoal. The bound counts were assayed for PGE\(_2\) and PGF\(_{2\alpha}\) by radioimmunoassay.

Figure 1. Separation of \(^3\)H-prostaglandin A\(_2\), E\(_2\) and F\(_{2\alpha}\) by silicic acid (Sigma, SIL-200B) column chromatography.

Table 1. Cross-Reactivity of Prostaglandin E\(_2\) and F\(_{2\alpha}\) Antisera with Known Prostaglandins

<table>
<thead>
<tr>
<th>Prostaglandin</th>
<th>Anti-PGE(_2) (1:8,000)</th>
<th>Anti-PGF(_{2\alpha}) (1:30,000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGF(_{2\alpha})</td>
<td>0.20</td>
<td>0.80</td>
</tr>
<tr>
<td>PGF(_{1\alpha})</td>
<td>0.01</td>
<td>0.20</td>
</tr>
<tr>
<td>PGF(_{2\alpha})</td>
<td>0.70</td>
<td>100.00</td>
</tr>
<tr>
<td>PGE(_2)</td>
<td>1.00</td>
<td>0.27</td>
</tr>
<tr>
<td>PGE(_1)</td>
<td>14.00</td>
<td>0.30</td>
</tr>
<tr>
<td>15-keto-PGE(_2)</td>
<td>0.01</td>
<td>0.70</td>
</tr>
</tbody>
</table>
determined by liquid scintillation spectrometry (Beckman Instruments), corrected for recoveries, and the results expressed as pg/10^6 cells/hr. The sensitivity of the assay was <5 pg/tube and the recoveries averaged 85%. Also, the interassay and intraassay variability was 9% and 6% respectively.

Since incubation of the cells with \(^3\)H-PGE\(_2\) or \(^3\)H-PGF\(_{2a}\) for periods of 1 and 2 hours failed to demonstrate metabolism or degradation of the prostaglandins, the levels of PGE\(_2\) and PGF\(_{2a}\) measured in the incubation media must reflect adrenal cell synthesis and release only.

Prostaglandin H\(_2\) was biosynthesized by the method of Green et al. using ram seminal vesicle microsomes. The product was identified as PGH\(_2\) by the following criteria: 1) treatment with stanous chloride converted it to a compound that behaved as PGF\(_{3a}\) by chromatographic and immunoassay methods; 2) treatment with bovine serum albumin converted it to a compound with the chromatographic properties of PGD\(_{2}\); and 3) when added to human platelets, it was converted to immunoreactive thromboxane B\(_2\). The PGH\(_2\) was stored at -20°C in dry diethyl ether. This ethereal solution was added to the adrenal cells during incubation at 37°C, and the diethyl ether vehicle was added to the control cells.

Statistical analyses were performed by analysis of variance and student's t test when comparing groups and by analysis of covariance when comparing dose-response curves.

**Results**

The effects of angiotensin II and III on the release of aldosterone and prostaglandins from adrenal cells is illustrated in figures 2 and 3. Both peptides caused a dose-related increase in aldosterone release without significantly altering the release of PGE\(_2\) or PGF\(_{2a}\). At the 10 pmoles/m at of angiotensin II, the PGE\(_2\) concentration in the medium was significantly elevated; however, with a higher dose, the PGE\(_2\) concentration returned to control levels.

Indomethacin was found to inhibit basal and angiotensin II-induced aldosterone release by 40% \((p < 0.001)\) and 15% \((p < 0.05)\) respectively (fig. 4). While angiotensin II failed to alter PGE\(_2\) release in the dose tested, indomethacin reduced the release of PGE\(_2\) into the medium by 60% in both control and angiotensin II treated cells. Similar results were obtained with angiotensin III with indomethacin inhibiting angiotensin III-stimulated steroidogenesis by 52% \((p < 0.01)\) and PGE\(_2\) release by 60% \((p < 0.05)\) (fig. 5). When figures 4 and 5 are compared, it is clear that indomethacin was more effective in inhibiting angiotensin III-induced steroidogenesis when compared with angiotensin II despite similar reductions in PGE\(_2\) release.

While angiotensin II did not release PGE\(_2\), it was possible that basal amounts of PGE\(_2\) might "prime" or sensitize the adrenal cortex to angiotensin II and that such an action could explain the inhibitory effects of indomethacin. However, when the effect of angiotensin II on aldosterone release was examined in the presence and absence of PGE\(_2\) in indomethacin-treated cells, similar dose responses were observed in both groups indicating a lack of sensitization by PGE\(_2\) (fig. 6). Furthermore, the effects of angiotensin II and PGE\(_2\) were additive only at the lowest dose of angiotensin II tested.

![Figure 2](http://hyper.ahajournals.org/DownloadedFrom)
Figure 3. Effect of angiotensin III on the release of aldosterone (left) and PGE$_2$ and PGF$_{2\alpha}$ (right) in rat adrenal cell suspensions. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with control.

Figure 4. Effect of indomethacin on angiotensin II-induced aldosterone release and prostaglandin release in rat adrenal cell suspensions. Each value represents the mean ± SEM. Statistical significance is indicated in the brackets connecting the compared bars.

Figure 5. Effect of indomethacin on angiotensin III-induced aldosterone release and prostaglandin release in rat adrenal cell suspensions. Each value represents the mean ± SEM. Statistical significance is indicated in the brackets connecting the compared bars.
To further examine the role of prostaglandins in steroidogenesis, the effects of the fatty acid precursor of PGE₂, arachidonic acid, was tested (fig. 7). While arachidonic acid caused a dose-related increase in PGE₂ and PGF₂α release, the fatty acid failed to alter the release of aldosterone. Similarly, the cyclic endoperoxide intermediate, PGH₂, increased the adrenal PGE₂ production by 230-fold, yet did not alter steroidogenesis (fig. 8).

**Discussion**

Our present experiments indicate that prostaglandin E₅ does not function as a mediator of adrenal steroidogenesis in physiologically relevant doses. While adrenal cortical cells are capable of synthesizing PGE₅ from both arachidonic acid and PGH₂, aldosterone release is not stimulated by these prostaglandin E₅ precursors. Other investigators have reported a dose-related increase in aldosterone release in vitro with prostaglandin E₂ and E₅; however, doses in the microgram range were required for steroidogenic activity. Thus, it appears that prostaglandin E₅ is unable to stimulate aldosterone
steroidogenesis is not stimulated by either arachidonic
angiotensin II and III do not stimulate prostaglandin
mediation or modulate basal or angiotensin-stimulated
cyclooxygenase and isomerase necessary for the syn-
thesis of PGE\textsubscript{2}, the failure of angiotensin to release the
prostaglandin must be due to a failure of the peptides
to release arachidonic acid from its phospholipid or
cholesterol ester stores. This lack of stimulation of the
angiotensin-sensitive phospholipase could be due to
the presence of glucocorticoids in the incubation
media since these steroids are known to inhibit phospholipase A\textsubscript{2}.\textsuperscript{14, 15} However, when steroid syn-
thesis is blocked by aminogluthimide, angiotensin II
still fails to stimulate the release of PGE\textsubscript{2} from
adrenal cells (M. J. Dunn, personal communication).
Thus, the mechanism of adrenal insensitivity to
angiotensin is unknown.

As in our previous studies,\textsuperscript{1} indomethacin inhibited
basal, angiotensin II, and angiotensin III-stimulated
aldosterone release as well as inhibiting the adrenal
synthesis of PGE\textsubscript{2} by 60%. Interestingly, indomethacin was more effective in inhibiting angioten-
sin III-induced steroidogenesis than that stimulated
by angiotensin II; however, the reason for this
difference is unknown. Indomethacin has also been
found to inhibit angiotensin-induced steroidogenesis
in normal subjects\textsuperscript{18} but not in patients with post-
malignant hypertension.\textsuperscript{19} This 60% decrease in basal
prostaglandin synthesis with indomethacin may be
interpreted to explain the observed decreases in basal
and angiotensin-stimulated aldosterone release.
However, since a dose of PGE\textsubscript{2} (100 ng) that was 250
times greater than the basal adrenal cell production
was required to stimulate aldosterone production
twofold in indomethacin-treated adrenal cells, this
would not appear to be the case. Furthermore, we
could not demonstrate that this dose of PGE\textsubscript{2} was ad-
ditive with angiotensin II on steroidogenesis or that it
sensitized the adrenal to the actions of angiotensin II.

These findings suggest that indomethacin inhibits
alcohol or release of an action independent of prostaglandin synthesis inhibition. Alternatively, indomethacin may inhibit angiotensin-steroidogenesis by blocking the synthesis of an arachidonic acid metabo-
lite other than prostaglandin E\textsubscript{2}. The most
probable prostaglandin candidate would appear to be
prostacyclin, PGI\textsubscript{2}. In this regard, Ellis et al.\textsuperscript{18} found
that PGI\textsubscript{2} was 100–1000 times more potent than PGE\textsubscript{2}
in stimulating the release of corticosterone from
adrenal cortical cells. However, this latter possibility
would appear unlikely in view of our findings that
steroidogenesis is not stimulated by either arachidonic
acid or PGI\textsubscript{2}.

In summary, prostaglandin E\textsubscript{2} does not appear to
mediate or modulate basal or angiotensin-stimulated
steroidogenesis. This is based on the observations that
angiotensin II and III do not stimulate prostaglandin
synthesis in steroidogenic doses, that supraphysiologi-
cal doses of PGE\textsubscript{2} are required for steroidogenesis,
and that arachidonic acid and PGI\textsubscript{2} fail to stimulate
steroidogenesis even though prostaglandin synthesis is
increased.

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