Angiotensin II Inhibits Luteinizing Hormone-stimulated Cholesterol Side Chain Cleavage Expression and Stimulates Basic Fibroblast Growth Factor Expression in Bovine Luteal Cells in Primary Culture*

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Angiotensin II has been identified immunohistochemically in the ovaries of both rats and humans. Here we present evidence that angiotensin II (an extremely vasoactive agent in a wide range of tissues) may be involved in the regulation of the major steroidogenic vasoactive agent in a wide range of tissues) may be involved in the regulation of the major steroidogenic enzyme in the ovary, cholesterol side chain cleavage cytochrome P-450 (P-450\textsubscript{scc}), as well as of basic fibroblast growth factor (bFGF), which has been implicated as an angiogenic factor in the bovine corpus luteum. We have used primary cultures of bovine luteal cells to examine the effect of angiotensin II and its receptor antagonist, saralasin, on expression of mRNA encoding bFGF, which has been implicated as an angiogenic factor in the bovine corpus luteum. We have used primary cultures of bovine luteal cells to examine the effect of angiotensin II and its receptor antagonist, saralasin, on expression of mRNA encoding bFGF, which has been implicated as an angiogenic factor in the bovine corpus luteum. We have used primary cultures of bovine luteal cells to examine the effect of angiotensin II and its receptor antagonist, saralasin, on expression of mRNA encoding bFGF, which has been implicated as an angiogenic factor in the bovine corpus luteum. We have used primary cultures of bovine luteal cells to examine the effect of angiotensin II and its receptor antagonist, saralasin, on expression of mRNA encoding bFGF, which has been implicated as an angiogenic factor in the bovine corpus luteum.

Luteinizing hormone (LH) caused a 15-fold increase in progesterone accumulation after 24 h of exposure which was reduced to 5-fold in the presence of angiotensin II. This appeared to be receptor-mediated in that although saralasin alone had no effect on LH-stimulated progesterone accumulation, it significantly reversed the inhibition by angiotensin II. This pattern was mirrored by the levels of mRNA encoding P-450\textsubscript{scc}, i.e., LH-induced the highest levels of expression of this message, these levels were reduced by angiotensin II, and saralasin partially overcame this reduction. Levels of mRNA encoding bFGF were elevated by both LH and angiotensin II. Treatment with saralasin, however, resulted in complete inhibition of bFGF mRNA expression in the presence of both LH and angiotensin II. These results suggest a role for angiotensin II to mediate the action of LH as a regulator of bFGF expression and hence, potentially, angiogenesis. Local production of angiotensin II might also contribute to the refractoriness of luteal progesterone secretion to LH at the time of luteal regression.

The role of the systemic renin-angiotensin system in cardiovascular fluid and electrolyte homeostasis has been well documented (Laraghet et al., 1972; Magness et al., 1987). Recently, a number of local renin-angiotensin systems have been identified as alternative sources of angiotensin II and postulated to be involved in local regulatory mechanisms (Mendelsohn, 1982; Ganong 1984; Pratt et al., 1984; Deschepper et al., 1986). There is growing evidence that the ovary is the site of such a locally active renin-angiotensin system.

Components of this local renin-angiotensin system have been identified in the ovaries of a number of species. Human follicular fluid contains prorenin- and renin-like activities as well as angiotensin II and angiotensin III (Fernandez et al., 1985; Culler et al., 1986; Lightman et al., 1987; Palumbo et al., 1989). The concentrations of these components of the renin-angiotensin system are significantly higher in follicles stimulated as part of an in vitro fertilization protocol as well as in unstimulated follicles in the preovulatory period (days 13-14) than in peripheral plasma (Fernandez et al., 1985; Lightman et al., 1987). Angiotensin II is present in the rat ovary in significantly higher levels than plasma (Husain et al., 1987), angiotensin II binding sites have been identified in rat ovarian follicles (Speth et al., 1986; Husain et al., 1987), and angiotensin-converting enzyme is present in some rat ovarian follicles and corpora lutea (Speth and Husain, 1988). More recently, both renin- and prorenin-like activities have been identified in bovine follicles (Schultzke et al., 1989).

The possible physiological importance of a local renin-angiotensin system remains unclear. Most recent research on the ovarian renin-angiotensin system has focussed on the involvement of this system in follicular steroidogenesis and ovulation. Angiotensin II increases estrogen secretion by rat ovarian tissue in vitro stimulated by pregnant mare serum gonadotropin (Husain et al., 1986; Pucell et al., 1987) and has been reported to increase choric gonadotropin-stimulated progesterone production in luteinized human granulosa cells (Paulson et al., 1989). A local renin-angiotensin system has also been implicated in ovulation (Pellicer et al., 1988), and the angiogenic process occurring during the ovarian cycle (Fernandez et al., 1985).

In order to examine the effects of angiotensin II and its receptor antagonist, saralasin, on bovine luteal cell function and given the reported stimulation by angiotensin II of ovarian steroidogenesis, we have examined its effects on progesterone production and the expression of mRNA encoding cholesterol side chain cleavage cytochrome P-450 (P-450\textsubscript{ccc})\textsuperscript{1} by bovine luteal cells in primary monolayer culture. Additionally, in view of reports that angiotensin II may be angiogenic, we have examined its effects on the expression of mRNA encoding basic fibroblast growth factor (bFGF), a

\textsuperscript{1}The abbreviations used are: P-450\textsubscript{ccc}, cholesterol side chain cleavage cytochrome P-450; bFGF, basic fibroblast growth factor; LH, luteinizing hormone.
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Materials and Methods

Bovine ovaries were obtained from non-pregnant cows at slaughter and transported to the laboratory in ice-cold McCoy's 5A culture medium. Corpora lutea were classified into four groups on the basis of gross morphology according to the method of Ireland et al. (1980). Stage II and III corpora lutea, corresponding to the early to midstages of the luteal phase, were sliced on a Stadie-Riggs band microtome and digested in McCoy's 5A culture medium containing 0.2% collagenase and 2.5% fetal bovine serum. This digestion was carried out in a shaking water bath at 37 °C for 3 x 40 min. At the end of each 40-min incubation, the supernatant was removed and the remaining tissue incubated with fresh medium and enzymes. The first supernatant was discarded, the cells from the second two incubations being collected by centrifugation, washed in collagenase-free medium, pooled, plated at a density of 10^6 cells/ml, and incubated overnight at 37 °C (95% air, 5% CO2). After an overnight incubation, cells that had attached to the culture dishes and were washed for 5-6 h in serum-free McCoy's 5A medium. Cells were then incubated for 24 h in serum-free medium with or without bovine luteinizing hormone (LH) (10 ng/ml), angiotensin II (10^{-11}-10^{-8} M), and saralasin (10^{-7} M). At the end of this incubation period medium was removed for progesterone assay, and the cells were either lysed in 0.5 M NaOH and assayed for protein using the method of Lowry et al. (1951) or scraped from culture dishes into 4 M guanidinium thiocyanate solution for RNA preparation (MacDonald et al., 1987).

RNA samples were subjected to electrophoresis through 1.1% agarose gels containing formaldehyde (6%). The RNA was then electroblotted to nylon membranes. Blots were then prehybridized and probed with either a 32P-labeled cDNA specific for bovine P-450, or a 32P-labeled RNA probe complementary to RNA encoding bFGF. These reactions utilized T7 RNA polymerase and were carried out in the presence of 40 mM Tris-HCl (pH 8), 8 mM MgCl2, 2 mM spermidine, 50 mM NaCl, 400 μM of each cold rNTP, and 50 nCi of [α-32P]UTP (800 Ci/mmol). Hybridizations were carried out at 65 °C in the same buffer as prehybridizations but containing 1 x 10^6 cpm/ml denatured labeled cRNA. Blots were then washed twice in 2 x SSC; 0.1% sodium dodecyl sulfate for 20 min each and twice in 0.1 x SSC; 0.1% sodium dodecyl sulfate for 15 min each, again at 65 °C. They were then blotted dry, wrapped in plastic film, and subjected to autoradiography.

Results and Discussion

Fig. 1 demonstrates the effects of angiotensin II and its receptor antagonist saralasin on progesterone production by unstimulated bovine luteal cells and cells stimulated with LH. Neither angiotensin II nor saralasin alone had any effect on progesterone production. This observation is consistent with a recent report that angiotensin II in the absence of gonadotropins had no effect on steroidogenesis in luteinized human granulosa cells (Paulson et al., 1989). Earlier work by Palumbo and co-workers (Palumbo et al., 1989), however, reported a direct effect of high doses of angiotensin II on estradiol, testosterone, and progesterone secretion, again using luteinized granulosa cells obtained from human menopausal gonadotropin/human chorionic gonadotropin-stimulated ovaries. These discrepancies are probably due to differences in in vitro fertilization treatment regimes and in vitro culture conditions and emphasize the usefulness of a suitable animal model for such studies.

In the present study, LH caused a marked increase in progesterone accumulation by cultured bovine luteal cells, which was greatly attenuated in the presence of angiotensin II. This effect appeared to be receptor-mediated, as it could be partially overcome by saralasin. These observations contrast with previous reports that angiotensin II increases LH-stimulated progesterone production in human granulosa cells (Palumbo et al., 1989; Paulson et al., 1989). This may represent either a species difference or a difference between luteinized granulosa cells and true luteal cells. The effects on progesterone accumulation in the present study appeared to be mediated at least in part by changes in levels of expression of the gene encoding P-450. (Fig. 2). Levels of mRNA encoding this enzyme closely mirrored progesterone production; angiotensin II significantly decreased LH-stimulated expression of mRNA encoding P-450, and this inhibitory effect of angiotensin II was blocked by the angiotensin II receptor antagonist, saralasin (Fig. 2).

Transcription of the gene encoding P-450 was influenced by a number of factors; gonadotropins stimulate transcription via cAMP-mediated mechanisms (Trzeciak et al., 1986), whereas activators of protein kinase C such as tetradecanoic acid stimulate this transcription in rat granulosa cells (Trzeciak et al., 1987). The events which mediate angiotensin II action subsequent to binding to its receptor are still poorly characterized; however, rapid alterations in intracellular free calcium ion concentrations occur in adrenal glomerulosa, adrenomedullary, and ovarian granulosa cells (Catt et al., 1987; Conner et al., 1987; Zimlichman et al., 1987) as well as formation of inositol phosphates (Gallo-Payat et al., 1986; Rossier et al., 1988; Bauks et al., 1988). Thus it seems possible that the effects of angiotensin II on LH-stimulated expression of P-450 are mediated at least in part by changes in intracellular free calcium ion concentrations.
of mRNA encoding P-450 \textsubscript{ccc} may be mediated, at least in part, by protein kinase C-dependent mechanisms.

Levels of mRNA encoding bFGF were increased by angiotensin II in a dose-dependent manner (Fig. 3). This increase appeared to be receptor-mediated, as it could be overcome by saralasin (Fig. 3). In addition, we confirmed previous observations that levels of mRNA encoding bFGF could be increased by LH (Fig. 4; Stirling, et al., 1989), suggesting that the gene encoding bFGF can be transcriptionally activated by factors whose action is mediated by protein kinase A. However, saralasin alone reduced expression of mRNA encoding bFGF to below control levels (Fig. 3 and 4), suggesting that endogenous angiotensin II stimulation in the cell cultures. Furthermore, saralasin also prevented the increase in this expression following LH treatment (Fig. 4), suggesting that LH may exert this effect in part via angiotensin II. Thus, a hypothesis is presented that LH may cause increased secretion of angiotensin II from luteal cells, which then in an autocrine or paracrine fashion increases the expression of mRNA encoding bFGF.

In conclusion, we have demonstrated effects of angiotensin II and its receptor antagonist saralasin, which suggest that angiotensin II is exerting complex and coordinated control on at least two distinct aspects of luteal function, expression of a steroidogenic enzyme, P-450 \textsubscript{ccc}, and a proposed angiogenic factor, bFGF. We have previously demonstrated that bFGF mRNA is expressed throughout the luteal cycle (Stirling et al., 1989). Perhaps angiotensin II produced early in the luteal phase stimulates bFGF production and hence the rapid angiogenesis occurring at that time. Local production of angiotensin II in terms of progesterone production and expression of P-450 \textsubscript{ccc} at the time of luteal regression. It seems clear from these studies that the potential for the involvement of angiotensin II as an intraovarian regulator of ovarian function is not limited to the developing follicle. Angiotensin II may be critically involved in the regulation of growth, development, and steroidogenic capacity of the bovine corpus luteum.

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