Control of adrenal cell proliferation by AT$_1$ receptors in response to angiotensin II and low-sodium diet

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Control of adrenal cell proliferation by AT$_1$ receptors in response to angiotensin II and low-sodium diet. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E303–E309, 1999.—The effects of angiotensin II (ANG II), the angiotensin type 1 (AT$_1$) receptor antagonist losartan, and low-sodium diet on rat adrenal cell proliferation were studied in vivo with immunocytochemistry. Both ANG II and low-sodium diet increased proliferation of endothelial cells of the zona glomerulosa. Losartan prevented ANG II-induced hyperplasia of glomerulosa cells but not the effects of a low-sodium diet. Glomerulosa cells after ANG II + losartan treatment appeared hypertrophied compared with those of controls. Proliferative effects of ANG II and low-sodium diet in the reticularis were blocked by losartan. No changes were seen in the fasciculata. Proliferation in the medulla was increased with losartan, was decreased by ANG II, but was unaffected by low-sodium diet. In conclusion, 1) cell hypertrophy and proliferation of glomerulosa cells are mediated by AT$_1$ receptor-dependent and -independent processes, 2) proliferation of reticularis cells is controlled by AT$_1$ receptors, and 3) reciprocal control of chromaffin cell proliferation by ANG II may involve indirect AT$_1$-dependent processes.

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In a previous study, we showed that long-term treatment with ANG II or a low-sodium diet increased cell proliferation in the zona glomerulosa of the rat adrenal gland more than twofold (19). Interestingly, neither treatment with ANG II nor a sodium-restricted diet had any effect on proliferation within the zona fasciculata, despite evidence of ANG II-induced steroidogenesis and growth in fasciculata cells of other species (4, 9, 25). However, an increase in cell proliferation was observed in a discrete population of cells within the zona reticularis. These cells of undefined steroidogenic function proliferated at the interface with the adrenal medulla. Within the medulla, proliferation of chromaffin cells decreased in response to ANG II but was unaffected by dietary sodium. A number of questions are raised by these observations, namely, 1) is cell proliferation controlled by the same type of ANG II receptor in glomerulosa and reticularis cell populations? 2) are the effects of dietary sodium mediated entirely by ANG II? and 3) are the antiproliferative effects of ANG II in the medulla also receptor mediated? These questions define the subjects of the present investigations.

Materials and Methods

Groups of eight adult male Wistar rats (Harlan Olac, Bicester, UK) weighing 200–250 g were housed in a temperature- and light-controlled room (21°C; 12:12-h light-dark cycle) and, before treatment, were given normal rat chow (Special Diets Services, Witham, Essex, UK) and water ad libitum. 5-Bromo-2′-deoxyuridine (BrdU) (Sigma, Poole, Dorset, UK), dissolved in a 1:1 (vol/vol) mixture of dimethyl sulfoxide and 0.154 M NaCl, was administered via miniosmotic pumps (model 2002, Alzet, Palo Alto, CA) to all rats. Losartan, a gift from Merck Sharp & Dohme, was administered in the drinking water at a concentration of 75 mg losartan/l (equivalent to a daily dose of ~2.25 mg/rat).
Experimental Groups

Under sodium pentobarbitone anesthesia, rats were implanted with miniosmotic pumps that infused BrdU (1.25 mg/day) continuously during experimental and control treatments. Two separate experiments were carried out.

ANG II infusion. Four groups of rats were investigated: 1) a control (untreated) group were given tap water to drink; 2) ANG II-treated rats were given a second miniosmotic pump with 6 mg/ml ANG II (200 ng·kg⁻¹·min⁻¹) and were given water to drink; 3) losartan-treated rats were given a solution of losartan to drink; and 4) ANG II + losartan-treated rats were infused with ANG II and given losartan solution to drink. Blood pressure was measured by tail cuff plethysmography (8).

Dietary sodium intake. Four groups of rats were given either 1) normal rat chow with water to drink, 2) low-sodium chow (10 mM Na⁺, 182 mM K⁺; Special Diet Services) and water to drink, 3) normal rat chow with losartan solution to drink, or 4) losartan-treated rats were given a solution of losartan to drink.

At the end of 2 wk of treatment, all rats were killed by a blow to the back of the head followed by decapitation. Trunk blood was collected for measurement of plasma hormones. Adrenal glands were excised, cleared of fat, bisected, and fixed in Methacarn solution as described previously (19).

Immunocytochemistry

The technique for detecting BrdU in nuclei has been described previously (19). Briefly, radial, dewaxed, 3-µm sections of adrenal were incubated overnight at 4°C with an anti-BrdU monoclonal antibody (Europath, Bude, Cornwall, UK) and then were incubated with a secondary antibody conjugated with alkaline phosphatase (Dako, Bucks, UK) for 1 h at 22°C. BrdU was detected in cells with an alkaline phosphatase substrate and fuchsin red as a chromogen. Sections were counterstained with hematoxylin, dehydrated in alcohol, and mounted in DPX (Merck, Glasgow, UK).

Measurement of Plasma Hormones

Plasma aldosterone (DPC, Los Angeles, CA), corticosterone (14) concentrations, and renin activity (23) were measured by radioimmunoassay.

Quantification

All tissues were analyzed in a single-blind fashion in random sections. Epithelial and nonepithelial cells were counted separately in the zona glomerulosa, zona reticularis, and medulla as described previously (19). A BrdU index was calculated as the percentage of BrdU-positive cells of the total number of cells counted in each zone as follows: BrdU Index = (no. of stained nuclei/total no. of nuclei) × 100. Cell size was assessed as the number of cells per square millimeter. This was measured with an automated image analysis system (Seescan, Cambridge, UK) and a computerized camera frame of 6,380 µm². Cells were counted as the number of whole nuclei within each of four frames per adrenal.

Data Analysis

Data are presented as means ± SE and are compared by unpaired t-test.

RESULTS

Cell proliferation was seen mostly in epithelial cells of all regions of the cortex (Figs. 1 and 2). The zona glomerulosa was clearly delineated from the zona fasciculata by a layer of small cells 3 to 5 cells thick, which we and others refer to as the zona intermedia (7, 20) but which has been variously called the clear or white zone (absence of staining for aldosterone synthase and 11β-hydroxylase) (24) or the sudanophile zone (absence of lipid droplets) (2). The zona intermedia contained few BrdU-positive cells. Cell proliferation within the zona fasciculata was quite variable but was clearly more abundant in the outermost region at the interface with the zona intermedia and became progressively less toward the inner zona fasciculata. The overall pattern of distribution of BrdU-positive cells in the fasciculata was unaffected by any of the treatments. In general, cell proliferation in the zona reticularis appeared as clusters of BrdU-positive cells localized at the interface with the medulla (Fig. 2). In the medulla, BrdU-positive cells were generally parenchymal (chromaffin), endothelial, and sustentacular cells.

ANG II Infusion ± Losartan

Blood pressure at the end of treatment was significantly higher after ANG II treatment than for controls (191 ± 14 and 150 ± 7 mmHg, respectively) (n = 8; P < 0.05); losartan treatment prevented the response to ANG II (135 ± 9 and 130 ± 6 mmHg for the ANG II + losartan and losartan alone groups, respectively). ANG II suppressed plasma renin activity (PRA) and tended to increase plasma aldosterone concentration (but not significantly); losartan markedly stimulated PRA with or without concomitant ANG II (Fig. 3A). Losartan tended to reduce plasma concentrations of aldosterone in rats treated with ANG II, but this was not statistically significant. There were no significant effects of any treatment on plasma corticosterone concentrations.

Figure 1 shows the effects of various treatments on the distribution of BrdU-positive nuclei in the outer adrenal cortex; the BrdU indexes in epithelial cells are summarized in Fig. 4A. Proliferation of nonepithelial cells was not affected by ANG II or losartan in the zona glomerulosa, zona reticularis, or the medulla (data not shown). ANG II caused an increase in zona glomerulosa width, with a 2.4-fold increase in cell proliferation (Figs. 1 and 4A). Cell hypertrophy is suggested by a decrease in the number of cells per square millimeter from 12,246 ± 281 for controls to 11,713 ± 639 for ANG II, but this difference was not statistically significant. ANG II increased cell proliferation twofold in the zona reticularis and decreased proliferation of the parenchymal chromaffin cells in the medulla (Figs. 2 and 4A).

Losartan, when administered without ANG II, decreased zona glomerulosa cell proliferation by almost 50% (Figs. 1 and 4A). Cell proliferation was increased in the medulla and decreased in the zona reticularis by losartan treatment (Figs. 2 and 4).

Losartan also prevented the increase in zona glomerulosa cell proliferation caused by ANG II (Figs. 1 and 4A), although, despite reduced hyperplasia, the cells of the zona glomerulosa appeared to be hypertrophied. The number of cells per square millimeter of zona glomerulosa with ANG II + losartan (10,081 ± 923)
was less than in tissue of untreated control rats (12,246 ± 281; P < 0.05) but not significantly different from values after treatment with losartan (12,002 ± 467) or ANG II (11,713 ± 639) alone. In the reticularis, losartan prevented the changes in cell proliferation (Figs. 2 and 4A) caused by ANG II (P < 0.01).

**DISCUSSION**

Hypertrophy of the zona glomerulosa is a well-established response to in vivo treatment with ANG II and to dietary sodium restriction, and previous studies have shown it to be due to both cell hypertrophy and cell hyperplasia (17, 20, 36). With the use of losartan, we have shown that ANG II effects on proliferation in vivo are mediated by AT₁ receptors, and these results confirm in vitro studies (25, 38). The finding that losartan decreased glomerulosa cell proliferation in rats fed a control diet suggests that the tonic or basal control of cell turnover in vivo is mediated normally via the AT₁ receptor. Unexpectedly, we found that the zona glomerulosa remained hypertrophied in tissues of rats given losartan + ANG II, despite the fact that cell proliferation was reduced to levels below control values. In part, this may be explained by an increase in cell size. It would seem that hyperplasia is driven by an AT₁-dependent process and hypertrophy by an AT₁-independent process, although this contrasts with previous studies in vascular smooth muscle cells showing that both processes are mediated by AT₁ receptors (5, 11). There are, however, other interpretations of these data. One possibility is that secondary factor(s) from the adrenal medulla maintain glomerulosa cell hypertrophy in a paracrine fashion. For example, both peptide and catecholamine secretions from the adrenal medulla are thought to be important in maintaining...
steroidogenic function in the adrenal cortex (for a review see Nussdorfer, Ref. 27) and, as is clearly evident in the present experiments, losartan causes hyperplasia of the adrenal medulla. However, this theory is weakened because the effects of losartan alone on cell proliferation in the medulla are greater than those of ANG II + losartan combined.

Another possibility is that systemic factors specifically control zona glomerulosa function. In general, adrenocortical cell hypertrophy is associated with increased lipid storage (26). It is therefore possible that the combined influence of ANG II and losartan might produce glomerulosa cell hypertrophy by indirectly stimulating lipid uptake or synthesis. Alternatively, differences in cell size might reflect osmotic changes induced, perhaps, by changes in plasma potassium levels. In vitro studies have shown that small physiological changes in extracellular potassium concentration and osmolality induce significant changes in cell size (10). However, it is unclear whether these changes are sustained in vivo or, indeed, whether plasma potassium concentration or osmolality is affected by ANG II + losartan.

The effects of losartan alone differ in several respects from those of captopril (19, 20). Both treatments tended to reduce zona glomerulosa width. However, the effects of losartan appeared to be due to a decrease in proliferation, with no significant effects on cell size. In contrast, captopril had no effect on the BrdU index of zona glomerulosa cells, but cells appeared shrunken with pyknotic nuclei, which we interpreted as a sign of imminent cell death. Unlike losartan, captopril had no effect on epithelial cells of the zona reticularis. It is not clear whether differences between the effects of losartan and captopril reflect aspects of autocrine control of adrenocortical steroidogenesis by the renin-angiotensin system or whether there are differences in patterns of humoral factors that compensate for AT1 receptor and converting enzyme inhibition.

The pattern of response to losartan in rats fed a low-sodium diet differed in several key respects from that of ANG II-treated animals. Losartan failed to prevent the hyperplasia of glomerulosa cells and only partially reduced the increase in plasma aldosterone that a low-sodium diet caused. One interpretation of this observation is that the dose of losartan was insufficient to block the actions of elevated plasma ANG II levels. However, in the same gland, changes in the reticularis were blocked by this dose of losartan. Also, we have noted that changes in smooth muscle cell proliferation induced by dietary sodium restriction are very effectively blocked by this dose of losartan (21). Whereas these data do not exclude a role for AT1 receptors in the response to low-sodium diet, they indicate that compensatory changes of other trophic factors or the removal of a tonic inhibitory influence may also be involved. The lack of effect of dietary sodium manipulations on zona fasciculata cells excludes the involvement of a number of trophins, e.g., ACTH, arginine vasopressin, and norepinephrine, that

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**Fig. 2.** BrdU-positive nuclei (red) in inner cortex and medulla of adrenals from control rat (A), ANG II-infused rat (B), rat fed a low-sodium diet (C), losartan-treated rat (D), rat infused with ANG II and treated with losartan (E), and rat fed a low-sodium diet and treated with losartan (F) (×380). ZR, zona reticularis; M, medulla.
have been shown to increase cell proliferation in all cortical cell types. Other trophic factors, e.g., endothelin and α-melanocyte-stimulating hormone (18, 34, 41), do appear to be more specific to the glomerulosa and, if increased by dietary sodium restriction, could account for hyperplasia in the presence of losartan. Another likely candidate is plasma potassium concentration. The interaction between ANG II and potassium in the control of aldosterone synthesis is well established (40). A more recent study (30) has shown that in the absence of ANG II (in angiotensinogen knockout mice), increases in plasma aldosterone concentration and hypertrophy of the zona glomerulosa in response to

Fig. 3. Plasma hormone concentrations after 2-wk infusion with ANG II ± losartan treatment (A) and after feeding a low-sodium diet for 2 wk ± losartan treatment (B). Data are means ± SE; n = 6–8. *P < 0.05; **P < 0.01; and ***P < 0.001, with horizontal bars showing comparisons between groups.

Fig. 4. BrdU indexes (% of nuclei which are BrdU positive) in epithelial cells of zona glomerulosa, zona reticularis, and medulla of adrenal glands from rats after 2-wk infusion with ANG II ± losartan treatment (A) and feeding a low-sodium diet for 2 wk ± losartan treatment (B). Data are means ± SE; n = 6–8. *P < 0.05; **P < 0.01; and ***P < 0.001, with horizontal bars showing comparisons between groups.
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dietary sodium restriction are associated with increases in plasma potassium concentration.

Several studies have suggested that part of the aldosterone response to a low-sodium diet is mediated by the removal of a tonic inhibitory effect of dopamine (12, 35). Dopamine infusions counteract the increase in growth and secretory activity of the zona glomerulosa in response to low-sodium diet. Somatostatin and atrial natriuretic peptide are two hormones with similar effects to dopamine (32, 34). Their tonic influences may be lost in the response to dietary sodium restriction.

Changes in cell proliferation in the medulla are in some, but not all, respects counter to changes in the cortex and are therefore not mediated by the same AT₁-dependent mechanism. It could be argued, however, that ANG II has antiproliferative effects in the medulla that are mediated by AT₂ receptors. Others have described just such a mechanism in PC-12W and R3T3 cells (22, 39), and receptors in the medulla are predominantly of the AT₂ type (3, 13, 15, 32, 34, 37). The present data contradict this theory in two key respects. First, raised ANG II due to low-sodium diet, as opposed to exogenous infusion, does not have antiproliferative effects in the medulla. Second, losartan has proliferative effects in the medulla when one might predict either no effect (because AT₁, not AT₂ receptors, are targeted) or an antiproliferative effect (because of activation of the endogenous renin-angiotensin system). A more likely explanation, which reinforces previous conclusions (19, 20), is that chromaffin cell proliferation adapts to try and maintain normal blood pressure in the face of altered pressor and dilator influences. Thus proliferation is low when blood pressure is raised due to exogenous ANG II, unaffected when endogenous ANG II is raised by a low-sodium diet or when the pressor effects of ANG II are blocked by losartan, and increased when losartan is given alone.

The pattern of changes in cell proliferation in the inner zona reticularis is entirely in keeping with an AT₁-mediated process and are subtly different from changes in the glomerulosa. In the reticularis, the stimulatory effects of low-sodium diet and ANG II infusion are both blocked by losartan. The function of these inner zone cells is not known. Probably, they synthesize steroids because various immunocytochemical studies have identified a uniform distribution of steroidogenic enzymes in this region, but it is unlikely that they synthesize aldosterone because the key enzyme aldosterone synthase is located exclusively in the zona fasciculata/reticularis cells from bovine adrenal cortex via the AT₁ receptor subtype. Endocrinology 132: 2206–2212, 1993.


REFERENCES


