Determinants of cardiac fibrosis in experimental hypermineralocorticoid states

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Young, Morag, Geoff Head, and John Funder. Determinants of cardiac fibrosis in experimental hypermineralocorticoid states. Am. J. Physiol. 269 (Endocrinol. Metab. 32): E657–E662, 1995.—Uninephrectomized rats maintained on 1.0% NaCl to drink and infused with aldosterone (0.75 μg/h) for 8 wk have previously been shown to develop hypertension, cardiac hypertrophy, and cardiac fibrosis. In the present study we have shown that K+ supplementation (1.0% NaCl plus 0.4% KCl drinking solution) alters neither the interstitial nor the perivascular fibrotic response to mineralocorticoid treatment. Second, rats receiving 0.75 μg/h 9α-fluorocortisol, a mineralocorticoid and glucocorticoid agonist, respond with hypertension and cardiac fibrosis without cardiac hypertrophy. Finally, intracerebroventricular infusion of the mineralocorticoid receptor antagonist RU-28318 blocks blood pressure elevation, but not cardiac hypertrophy or fibrosis, when aldosterone is coinfused peripherally. We conclude that the myocardial fibrosis observed in response to chronic mineralocorticoid elevation and salt loading is a humorally mediated event independent of hypokalemia, hypertension, and cardiac hypertrophy. It remains to be determined whether the fibrosis observed in the presence of excess salt represents a direct (e.g., cardiac) effect of mineralocorticoid hormones or one mediated via a primary action on classical epithelial aldosterone target tissues (e.g., kidney).

aldosterone; deoxycorticosterone; 9α-fluorocortisol; hypokalemia; RU-28318; RU-38486

THE PHYSIOLOGICAL mineralocorticoid aldosterone classically acts on epithelial tissues such as the kidney and colon to promote unidirectional sodium transport and on circumventricular organs in the brain to stimulate salt appetite. Whereas mineralocorticoid receptors (MR) have been described in epithelial tissues, consistent with the physiological role of aldosterone in sodium homeostasis, they are also found in other tissues such as hippocampus and heart. MR levels are high in colon and hippocampus and low in kidney and heart (19), suggesting that MR abundance is independent of whether the tissue is epithelial or nonepithelial. In all four tissues, MR have essentially equivalent affinity for aldosterone, corticosterone, and cortisol (19).

Given their much higher circulating concentrations, glucocorticoids might be expected to occupy these intrinsically nonselective receptors. However, in kidney, colon, and parotid gland glucocorticoids are converted to their inactive 11-keto congeners by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-OHSD2), thus denying them access to MR; aldosterone is protected from similar dehydrogenation by the formation of a highly stable 11,18-hemiketal group (11, 13). In the hippocampus and heart, however, such “MR” do not appear to be protected by 11β-OHSD2, and presumably cannot discriminate in terms of occupancy between aldosterone and physiological glucocorticoids in vivo.

In this context Brilla and Weber (6) have demonstrated, in uninephrectomized rats drinking 1.0% NaCl solution, that infusion of aldosterone at a constant dose (0.75 μg/h sc) for 8 wk is followed by a marked accumulation of interstitial and, to a lesser extent, perivascular collagen in the heart. In subsequent studies (28) we have confirmed these findings and extended them in several ways. First, we found that deoxycorticosterone (DOC, 20 mg/wk sc) produced a similar degree of hypertension and cardiac hypertrophy to that seen with aldosterone, but commonly with less interstitial and more perivascular fibrosis.

Second, whereas administration of the physiological glucocorticoid corticosterone B (2 mg/day, sc) had little if any effect on blood pressure, heart weight, or collagen deposition, administration of the antiguclocorticoid, antiprogesterin RU-486 significantly elevated perivascular fibrosis. Given that DOC (in contrast with aldosterone) is also a glucocorticoid antagonist at the high doses used, we interpreted this as evidence for a possible mineralocorticoid agonist-glucocorticoid antagonist contribution to the genesis of perivascular cardiac fibrosis, and the possibility that under physiological circumstances glucocorticoid receptor occupancy by corticosterone acted as a tonic inhibitor of perivascular collagen deposition. Finally, although left ventricular wall thickness in aldosterone- and DOC-treated rats was substantially higher than in the other groups, consistent with their elevated blood pressure, no differences were seen in right ventricular wall thickness between groups. In contrast, the response in terms of interstitial collagen deposition was indistinguishable between left and right ventricles, supporting a humoral rather than hemodynamic etiology.

To further extend these studies we have undertaken three further series of steroid treatment experiments. First, given the hypokalemic action of continuous aldosterone or DOC administration, and the cardiotoxic effects of prolonged hypokalemia, we have compared the effects of these mineralocorticoids as previously given (i.e., with 1.0% NaCl to drink) with those animals similarly treated except that they were offered 1.0% NaCl plus 0.4% KCl as drinking fluid. Second, the possible mineralocorticoid agonist-glucocorticoid antagonist interaction in perivascular fibrosis was explored by studies on rats with both classes of adrenal steroids removed by adrenalectomy, in rats treated with the dual MR-glucocorticoid receptor agonist 9α-fluorocortisol, and in rats given aldosterone and RU-486 concurrently. Finally, the role of hypertension and cardiac hypertrophy in the etiology of the fibrotic response was explored by comparison of cardiac collagen deposition in rats made hypertensive by peripheral infusion of aldosterone.
and in rats in which the blood pressure elevating effect of aldosterone was blocked by concurrent intracerebroventricular (ICV) infusion of the selective MR antagonist RU-28318.

METHODS

Animal models. Male Sprague-Dawley rats (Baker Institute Animal House, Prahran, Victoria, Australia), initial weight 180–200 g, were uninephrectomized under Brielal Sodium (Lilly, Indianapolis IN, 55 mg/200 g ip) anesthesia and maintained on standard rat chow (Norco, Burnley, Australia) and a 1.0% NaCl drinking solution. The control group received no further treatment, whereas other groups received 1) bilateral adrenalectomy (Adrx, n = 8); 2) 9α-fluorocortisol (9α-F, Sigma, 0.75 μg/h sc for 8 wk, n = 8) via osmotic minipumps (Alzet, Palo Alto, CA); 3) DOC (Sigma Chemical, St. Louis, MO, 20 mg/day sc, n = 8); 4) aldosterone (Aldo, Sigma, 0.75 μg/h ivsm, 8 wk, n = 8); 5) RU-486 (Roussel-UCLAF, Romainville, France, 2 mg/day sc, n = 8); 6) aldosterone (0.75 μg/h ivsm for 8 wk) plus RU-486 (2 mg/day sc, 8 wk, Aldo + RU); 7) aldosterone (0.75 μg/h ivsm for 8 wk) plus isotonic saline (infused into the left cerebral ventricle via a permanent indwelling cannula from osmotic minipumps, n = 8; ICV-C); 8) aldosterone (0.75 μg/h ivsm for 8 wk) plus RU-28318 (Roussel-UCLAF, 1.1 μg/h infused into the left cerebral ventricle for 8 wk, n = 8; ICV-RU); 9) aldosterone (0.75 μg/h ivsm for 8 wk) with 0.1% KCl (included in the 1.0% NaCl drinking solution, n = 8; Aldo + K); 10) DOC (20 mg/wk sc) with 0.1% KCl (included in the 1.0% NaCl drinking solution, n = 8; DOC + K).

Rats receiving an infusion in the left cerebral ventricle were implanted with a stainless steel catheter into the lateral cerebral ventricle (4) at the same time as uninephrectomy. The cannula (28-gauge C313-I, Plastic Products, Roanoke, VA) was implanted with dental cement, and the skin sutured together covering the cannula. The end was connected to vinyl tubing, which was buried subcutaneously and attached to the osmotic minipumps, so that the infusion system was not exposed and animals could be housed in groups.

Throughout the 8-wk study, weekly systolic blood pressure measurements (tail-cuff method) and body weights were recorded. At the end of the treatment period the animals were anesthetized, the abdomen opened, and an arterial blood sample obtained from the abdominal aorta. The hearts were then excised, blotted, weighed, and divided in the midcoronal plane; the apex was snap frozen and stored for biochemical analysis, and the remaining heart tissue immersion-fixed in 4% buffered formaldehyde for histology.

Biochemical analysis. The concentration of hydroxyproline (OHP) in heart tissue was determined as described previously (9) as an estimate of tissue collagen content. The tissue was prepared for assay by overnight hydrolysis in 6 M HCl and subsequently made up to 12 ml with additional acid. Aliquots (200 μl) were dried and reconstituted with 50% isopropanol for assay. OHP content of the samples was expressed as mg OHP/g dry tissue.

Morphological analysis. Fixed tissue blocks from each heart were paraffin-embedded and sectioned at 3 μm. After being deparaffinized and rehydrated, the sections were stained for 1 h with 0.1% Sirius Red (Polysciences, Warrington, PA) in saturated aqueous picric acid, rapidly dehydrated for 2 × 1 min in 100% ethanol and mounted in Depex (BDH Chemicals, Port Fairv, Australia) (6).

To determine the collagen volume fraction, the stained area of the tissue was measured for each sampled field and expressed as a proportion of the total area within each field. One section from each heart was analyzed at 16 × objective magnification under green filtered light. Images gathered with a charged-coupled device video camera were digitized and reference dark and light fields, respectively, subtracted and divided from the image to correct for uneven background illumination. A gray level threshold was set for stained collagen, and the area stained calculated as a percentage of the total area (Bioscan Optimas, Edmonds, WA) (27).

The sections were sampled blind by a single observer in a systematic fashion (18). For analysis of left and right ventricular interstitial collage, every fifth field was analyzed as the ventricle was scanned. Fields containing vessels or artifacts were not included. A similar method was used to sample perivascular collagen: in this case only those fields containing vessels were considered. In each ventricle 15–16 fields without artifacts (folds, splits, etc.) were analyzed for interstitial collagen. For quantification of perivascular collagen only those fields containing a blood vessel (2–3 fields per heart) were analyzed. An estimate of the change in perivascular collagen content between groups was determined by measuring the total collagen present in sampled fields containing vessels and correcting for interstitial collagen.

Statistical analysis. To address the three questions outlined above, the following between-group comparisons were made: 1) control, Aldo, Aldo + K, DOC, DOC + K; 2) control, Adrx, 9α-F, Aldo, RU-486, Aldo + RU; and 3) control, ICV-C, ICV-RU.

One-way analysis of variance was done on all data sets for each of these three groups (SPSS/PC, Information Analysis Systems, SPSS, Chicago, IL). In addition, the Duncan test for multiple comparisons was applied to all data sets to test for statistical significance (16), and the null hypothesis was rejected at P < 0.05.

RESULTS

The possible etiologic role of hypokalemia in mineralocorticoid salt-induced cardiac fibrosis. KCl supplementation gave plasma K⁺ concentration values not different from control (3.8 ± 0.3 meq/l) for both aldosterone plus K⁺ (3.1 ± 0.4 meq/l) and DOC plus K⁺ groups (3.5 ± 0.2 meq/l); in contrast, values for aldosterone alone (1.9 ± 0.1 meq/l) and DOC alone (2.7 ± 0.2 meq/l) were significantly (P < 0.01) lower than both control and the equivalent K⁺-supplemented group. KCl supplementation did not, however, consistently blunt the mineralocorticoid-induced effects on the various indexes measured, as shown in Fig. 1. In terms of final systolic blood pressure (Fig. 1A), there is clearly no difference between groups with and without K⁺; similarly, K⁺ administration does not lessen the extent of cardiac hypertrophy seen after administration of aldosterone or DOC (Fig. 1B); in fact, animals receiving DOC plus KCl had higher values than those receiving DOC alone (P < 0.05), reflecting body weight (DOC 417 ± 10 g, DOC + K⁺ 375 ± 22 g) rather than heart weight (1.39 ± 0.05 and 1.43 ± 0.05 g) differences. In terms of cardiac OHP content (Fig. 1C), K⁺ supplementation appears to be without effect in both the DOC and aldosterone groups.

In the left ventricle (Fig. 1D) K⁺ supplementation appears to significantly lower (P < 0.01) the aldoste-
steroids and cardiac fibrosis

Fig. 1. A: systolic blood pressure (SBP, mmHg) at 8 wk of treatment. B: heart weight (mg/body weight (g) as an index of cardiac hypertrophy (ICH). C: hydroxyproline concentration in the heart (mg OHP/g dry tissue). D and E: interstitial collagen volume fraction for left (LVIF) and right (RVIF) ventricles (percentage of total area). F: perivascular collagen fibers (PVF, arbitrary units). CON, control; DOC, deoxycorticosterone; DOC + K, deoxycorticosterone with potassium supplementation; Aldo, aldosterone; Aldo + K, aldosterone with potassium supplementation. Values are mean ± SE; n = 8 except for Aldo (n = 7). All values in treated rats were significantly different from control, at \( P < 0.01 \), except where indicated (NS or \( \dagger \); \( P < 0.05 \)). Values in treated animals with and without potassium supplementation were not significantly different, except where indicated (* \( P < 0.05 \); ** \( P < 0.01 \)).

aldostrone treatment was significantly more potent than DOC in stimulating interstitial collagen accumulation in both the left (\( P < 0.01 \)) and right (\( P < 0.05 \)) ventricles. Finally, for either mineralocorticoid, values for perivascular collagen (Fig. 1F) are clearly unaltered by KCl supplementation.

Mineralocorticoid agonist-glucocorticoid antagonist effects on perivascular fibrosis. In our previous study we interpreted the more marked effect of DOC (and of the antiglucocorticoid RU-486) on perivascular fibrosis as suggesting a possible mineralocorticoid agonist-glucocorticoid antagonist interaction as important for collagen deposition in this compartment, as opposed to the interstitium. To explore this question further we compared the effects of adrenal steroid withdrawal (adrenalectomy), administration of a potent mineralocorticoid agonist-glucocorticoid agonist (9α-fluorocortisol), and administration of aldosterone and RU-486 in combination.

As shown in Fig. 2A adrenalectomy is without effect on blood pressure: RU-486 alone elevates blood pressure modestly but significantly (\( P < 0.05 \)), with 9α-fluorocortisol and aldosterone (with and without RU-486) producing progressively more marked elevations (\( P < 0.01 \) vs. control). The final blood pressure with aldosterone alone was significantly higher (\( P < 0.01 \)) than for any other group. In terms of cardiac hypertrophy (Fig. 2B), no effect of adrenalectomy, RU-486, or 9α-fluorocortisol was seen. Aldosterone substantially and significantly (\( P < 0.01 \)) elevated heart weight above control; in contrast with blood pressure, no difference was noted between aldosterone alone and aldosterone plus RU-486. The lack of cardiac hypertrophy seen in the 9α-fluorocortisol group is not an artifact of a higher final body weight (control 448 ± 12 g, 9α-fluorocortisol 405 ± 15 g), in that heart weights were almost exactly proportional (1.33 ± 0.04, 1.16 ± 0.05).

Adrenalectomy modestly raised cardiac OHP levels (Fig. 2C), but was without effect on interstitial or perivascular collagen measured morphometrically (Fig. 2, D–F). RU-486 modestly elevated cardiac OHP, and both interstitial (Fig. 2, D and E) and perivascular collagen levels (Fig. 2F). The absence of cardiac hypertrophy notwithstanding, 9α-fluorocortisol administration was clearly followed by increased cardiac fibrosis, measured either as OHP content (Fig. 2C), interstitial collagen volume fraction in the left (Fig. 2D) or right (Fig. 2E) ventricle, or as perivascular collagen (Fig. 2F). Levels in the aldosterone group are similarly elevated, with RU-486 having an ameliorative effect on left ventricular interstitial collagen accumulation but not on the other indexes. In particular, there appears to be no increment in perivascular collagen in the aldosterone plus RU-486 group over aldosterone alone.

The role of systemic hypertension in mineralocorticoid-salt cardiac fibrosis. The third question addressed was that of the role of the elevated blood pressure in the peripheral cardiac response to aldosterone and a high-salt intake. As shown in Fig. 3, ICV infusion of the selective mineralocorticoid receptor antagonist RU-
**Fig. 2.** A–F: see Fig. 1, A–F. ADX, adrenalectomy; 9a, 9α-fluorocortisol. Values are means ± SE; n = 8 except for Aldo and Aldo + RU-486 (n = 7). A: treatment raised blood pressure (BP) significantly \((P < 0.01)\) except where indicated NS or \(*; P < 0.05; Aldo alone raised BP to levels higher \((P < 0.01)\) than other treatments. B: only Aldo, with or without RU-486, significantly \((P < 0.01)\) raised the ICH. C: all treatment groups showed elevation of OHP concentration above control \((P < 0.01)\), with 9a-F and Aldo alone also significantly higher \((P < 0.05)\) than ADX or RU-486 alone. D and E: all treatments raised interstitial collagen volume fraction \((P < 0.01)\) vs. control except where indicated (NS); 9a-F, Aldo + RU-486, and Aldo levels were also significantly \((P < 0.01)\) higher than those with ADX and RU-486. F: for PVF all treatments elevated \((P < 0.01)\) levels except where indicated NS; values for Aldo and 9a-F were also significantly \((P < 0.01)\) higher than those for RU-486 alone, as were those for Aldo + RU-486 \((P < 0.05)\).

**Fig. 3.** A–F: see Fig. 1, A–F. Periph, peripheral administration; ICV, intracerebroventricular administration. Values are means of: SE; n = 8 except for Aldo + RU-28318 \((n = 10)\). For both treatment groups \(P < 0.01\) vs. control except in A, where Aldo + NaCl is higher than control and Aldo + RU-28318, and Aldo + RU-28318 not different from control (NS).

**DISCUSSION**

The studies detailed in the present paper make three points. First, hypokalemia is not required for cardiac fibrosis.
fibrosis in response to mineralocorticoids plus salt. Second, although glucocorticoid antagonist activity may marginally increase perivascular fibrosis, in the present studies the effect is a subtle one and the predominant effect is MR mediated. Third, interstitial and perivascular fibrosis can be produced by mineralocorticoids and salt without substantial hypertension or cardiac hypertrophy.

Although K+ supplementation elevated plasma K+ concentration significantly above levels seen with mineralocorticoid administration alone, and no significant difference was found between levels in supplemented and control animals, this lack of significance may reflect the relatively large variance in the supplemented groups. Such increased variance has been previously reported (23) and ascribed to the length of time between lights on (i.e., cessation of feeding and drinking activity) and sampling, a time in our studies that varied between 3 and 6 h. This caveat notwithstanding, the striking lack of correlation between plasma K+ concentration and cardiac fibrosis, between aldosterone and DOC groups, and between supplemented and mineralocorticoid-alone groups, makes it difficult to ascribe a causative role to such hypokalemia in the process.

In terms of hypokalemia it has previously been suggested that chronic aldosterone administration to salt-loaded rats is not directly cardiotoxic, but that the induced hypokalemia is responsible for myocyte necrosis, in that it can be ameliorated by K+-sparing diuretics or K+ supplementation (8, 26). Subsequently, the time course of steroid and salt administration has been shown to be of considerable importance in terms of this hypokalemic effect, in that supplementation for 8 wk with 0.3% KCl showed comparable results to those in the present study (22), and 12 wk of treatment was required to reduce the incidence of microscopic, type III collagen scars (5) or of arrhythmias (17, 20).

The possible involvement of glucocorticoid receptor antagonism in the etiology of perivascular fibrosis was suggested by previous studies from this and other laboratories (6) comparing aldosterone and DOC, and by the effects we saw previously with RU-486 (28). In both previous studies (6, 28), the effect of DOC on perivascular fibrosis was higher than that of aldosterone, and that on interstitial fibrosis lower; the effects of two steroids are significantly different in the present study only when values are expressed as a ratio of collagen deposition in the two compartments (right ventricle P < 0.05, left ventricle P < 0.01). In contrast with our previous study, however, the perivascular response to aldosterone was a very marked (2.5-fold) increase. Although (as in the previous study) RU-486 modestly increased perivascular fibrosis, perhaps (as previously suggested) reflecting a physiological role for corticosterone as a tonic inhibitor of perivascular collagen accumulation, not surprisingly RU-486 and aldosterone administered together in the present study produced no increase over levels seen with aldosterone alone. In addition, the clear-cut effects of 9a-fluorocortisol on perivascular fibrosis, and the lack of effect of adrenalectomy, both argue against a necessary rule for glucocorticoids in preventing or ameliorating such a response.

In previous studies from Weber’s (25) laboratory low-dose spironolactone administration has been shown to prevent the effects of aldosterone on cardiac fibrosis without lowering blood pressure. The present studies extend this observation in a number of ways. First, when the blood pressure effects of peripheral aldosterone are antagonized by ICV infusion of RU-28318, the extent and pattern of cardiac fibrosis are unchanged, confirming the independence of fibrosis and blood pressure. Second, cardiac fibrosis in response to excess mineralocorticoid plus salt appears independent not only of hypertension but also of cardiac hypertrophy. Again, this has been suggested by the findings in previous studies, in that the extent of fibrosis in right and left ventricles was indistinguishable, despite a clear increase in left ventricular wall thickness in the aldosterone- and DOC-treated groups, and no difference in right ventricular wall thickness. What the present studies show is that the hypertrophic effect of administered aldosterone, previously shown to reflect increased left but not right ventricular wall thickness (6, 28), is independent of whether blood pressure rises (cf. Fig. 3, A and B). In addition, from the study with 9α-fluorocortisol, it is clear that a high level of cardiac fibrosis can be induced with no evidence for cardiac hypertrophy.

In mechanistic terms, then, mineralocorticoids appear to be acting on three separate, or at least experimentally separate, processes. First of these is blood pressure regulation, where mineralocorticoid receptors in the AV3V region (3), the septum (21), the area postrema (7), and in the paraventricular nucleus of the hypothalamus (24) have been implicated in the hypertensive response to mineralocorticoids. In a series of landmark studies Gomez-Sanchez and colleagues (13) have shown that whereas ICV infusion of aldosterone (10 ng/h) to the uninephrectomized rat raises blood pressure, infusion of similar doses (10–20 ng/h) of corticosterone does not mimic the effect of aldosterone, but antagonizes it in a dose-dependent fashion. Because the hypertensive response to aldosterone is antagonized by similar doses of administered corticosterone, the MR involved are presumably not protected from glucocorticoids by 11β-OHSD2, consistent with levels of mRNA for this recently cloned enzyme being below detection limits in brain extracts (1). In addition, they differ from epithelial MR where corticosterone and cortisol have been shown to mimic the action of aldosterone provided they can access the receptors (12), in that in the central receptors mediating the hypertensinogenic effects of aldosterone the physiological glucocorticoid occupies the receptor as an antagonist.

Second, aldosterone clearly has effects on cardiac hypertrophy, independent of blood pressure and mimicked by DOC, but not by 9α-fluorocortisol. This effect has been postulated to be direct (10, 15) or to involve release of an intermediary recognized to have cardiac trophic effects, for example angiotensin II or transforming growth factor-β (27). The site of action of aldosterone in this regard is unclear; as for the brain, levels of mRNA for 11β-OHSD2 are below detection limits in
infused with aldosterone await exploration. If the effect of peripherally administered mineralocorticoids is directly on cardiac MR, which on a tissue basis are found at levels comparable to those in kidney (2, 19), then such an action presumably reflects antagonism by aldosterone of a physiological effect of corticosterone, which normally tonically inhibits cardiac fibrosis through occupancy of MR by mechanisms yet to be explored.

In previous studies, the effects of administered aldosterone have been shown to be crucially dependent on the salt status of the animal (6). Rats on a restricted salt intake, infused with aldosterone at the standard dose level of 0.75 μg/ml had neither blood pressure nor cardiac fibrosis above control levels. How salt restriction protects against these effects of aldosterone is currently unclear, although recently paradoxical effects of sodium loading have been reported for components of the tissue renin-angiotensin system in vessel wall; whereas an elevated plasma volume decreases the activity of the circulating renin-angiotensin system, the converse is true at the tissue level, in that angiotensinogen levels and angiotensin-converting enzyme activity have been shown to be elevated in the aorta of rats maintained on a high-salt diet (29). The sensors mediating such changes and the mechanisms of their involvement in the cardiac hypertrophy and fibrosis in rats on a high-salt diet infused with aldosterone await exploration.

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