Mineralocorticoids, Hypertension, and Cardiac Fibrosis

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Abstract

Uninephrectomized rats drinking 1% sodium chloride were given aldosterone (Aldo, 0.75 μg/h, subcutaneous [s.c.] infusion), deoxycorticosterone (DOC, 20 mg/wk, s.c.), corticosterone (B, 2 mg/d, s.c.), or the antiglucocorticoid-antiprogestin RU486 (2 mg/d, s.c.) for 8 wk, and hemodynamic and tissue responses were compared with a non–steroid-treated control group. Aldo and DOC markedly increased systolic BP and caused considerable (40–50%) cardiac hypertrophy; B and RU486 caused neither hypertension nor cardiac hypertrophy. Measurements of ventricular cross-sectional areas showed hypertrophy due to an increase in mass of the left ventricle only. Cardiac hydroxyproline concentration was increased considerably by Aldo and DOC, to a lesser degree by RU486, and not by B. Aldo markedly elevated left ventricular interstitial collagen (2.5-fold vs control, $P < 0.01$ vs all groups); other steroid treatments also increased interstitial collagen over control (DOC × 1.8-, RU486 × 1.6-, B × 1.3-fold), with identical responses for right and left ventricles ($r = 0.94$). A different pattern of perivascular fibrosis was noted; DOC elevated perivascular collagen (2.1-fold vs control, $P < 0.01$ vs all other groups); RU486 raised levels 1.4-fold vs control, but neither Aldo nor B significantly affected perivascular collagen. These data are consistent with interstitial cardiac fibrosis reflecting type I (mineralocorticoid) receptor occupancy by administered Aldo or DOC, or by elevated endogenous B after type II (glucocorticoid) receptor blockade after RU486 administration; perivascular fibrosis may reflect a composite response after type I receptor agonist/type II glucocorticoid receptor antagonist occupancy. (*J. Clin. Invest.* 1994. 93:2578–2583.) Key words: interstitial fibrosis • perivascular fibrosis • aldosterone • deoxycorticosterone • RU486

Introduction

Aldosterone is secreted from the zona glomerulosa of the adrenal cortex in response to increased plasma levels of angiotensin II or potassium and acts on epithelial tissues to promote unidirectional sodium flux. Classically, its role in the maintenance of circulatory homeostasis is considered primarily to reflect its effect on plasma and extracellular volume, via renal salt and water retention. Recently, however, various extraepithelial roles for aldosterone have also been documented, a number of

which may also have cardiovascular effects. Salt appetite, for example, is increased by intracerebroventricular administration of aldosterone to rats (1); this effect is neither mimicked nor blocked by physiological doses of glucocorticoids, providing evidence for a specific extraepithelial effect of mineralocorticoids.

The specificity of mineralocorticoid action in aldosterone target tissues is enzyme rather than receptor based. Mineralocorticoid receptors in preparations of kidney or hippocampus, for example, bind aldosterone and the physiologic glucocorticoid cortisol (in rats, corticosterone) with equal affinity (2, 3). The circulating levels of glucocorticoids are very much higher than those of aldosterone; what enables aldosterone to occupy mineralocorticoid receptors in physiologic target tissues is the enzyme hydroxysteroid dehydrogenase (11βHSD),[1] which metabolizes cortisol and corticosterone to their receptor-inactive 11-keto congeners cortisone and 11-dehydrocorticosterone. Aldosterone is not similarly metabolized, as its hydroxyl at C11 is protected by cyclization with the unique, highly reactive aldehyde group at C18 to form a stable 11, 18 hemiketal (4, 5).

Whereas the mineralocorticoid receptors which mediate aldosterone action in epithelia (and, by extension, its effect on salt appetite) are protected by 11βHSD, those in other nonepithelial tissues, e.g., hippocampus and heart, clearly are not protected similarly. When adrenalectomized rats are injected with tritiated aldosterone or tritiated corticosterone, mineralocorticoid receptors in hippocampus and heart bind either tracer indistinguishably, in contrast with the marked aldosterone selectivity of kidney, colon, and parotid (4). Such "mineralocorticoid receptors" in nonprotected tissues are thus functionally high affinity receptors for corticosterone/cortisol, given the equivalent affinity and the much higher circulating levels of glucocorticoids. What this means, in turn, is that studies documenting extravascular effects of mineralocorticoids on tissues such as the heart may reflect pathophysiologic rather than physiologic actions of aldosterone.

Such effects on the heart were described recently by Brilla and Weber (6) in studies in which uninephrectomized rats drinking 1.0% NaCl responded to 8 wk of infusion of aldosterone by developing hypertension and interstitial cardiac fibrosis and to deoxycorticosterone (DOC) by developing hypertension and increased perivascular fibrosis. The effects of aldosterone on interstitial development of fibrosis were blocked by low- and high-dose spironolactone treatment, even though the lower dose did not reverse the hypertension (7). In addition, in vitro studies from the same laboratory have reported that aldosterone doubled the incorporation of tritiated hydroxyproline into collagen by isolated cardiac fibroblasts (8), consistent with a local rather than a systemic effect via mineralocorticoid receptors.

In light of these challenging data, we resolved to repeat and extend the studies from Weber's laboratory. In this report we addressed two issues in particular. First was the possibility that the cardiac fibrotic response may be nonspecific (i.e., equally produced by glucocorticoid or mineralocorticoid occupancy of mineralocorticoid receptors), given that such binding sites in the heart are clearly nonspecific. Second, we explored the dichotomy of aldosterone and deoxycorticosterone effects on perivascular fibrosis, in light of the glucocorticoid receptor antagonist activity of deoxycorticosterone, as well as its potent mineralocorticoid activity. The findings confirm those reported previously and in addition offer a conceptual framework of mineralocorticoid and glucocorticoid activity for further analysis of adrenal steroid effects on the heart.

Methods

Animal model. Five groups of male Sprague-Dawley rats (Monash Animal House, Clayton, Victoria, Australia), initial weight 180–200 g (mean±SEM), were uninephrectomized under Brietal sodium (Eli Lilly & Co., Indianapolis, IN; 55 mg/200 g, intraperitoneally) anesthesia. Rats were maintained on standard rat chow (Norco, Burnley, Victoria, Australia), sodium content 0.4–0.6%, and received 1% sodium chloride to drink. The control group was otherwise untreated while the other groups received (*a*) aldosterone (Sigma Immunochemicals, St. Louis, MO), 0.75 μg/h delivered subcutaneously (s.c.) via osmotic mini pumps (Alzet, Palo Alto, CA) for 8 wk (pumps changed at 4 wk); (*b*) deoxycorticosterone (Sigma Immunochemicals), 20 mg/wk s.c. injection; (*c*) corticosterone (Sigma Immunochemicals), 2 mg/d s.c. injection; and (*d*) RU486 (Roussel-UCLAF, Romainville, France), 2 mg/d s.c. injection. Each experimental group began the 8-wk study 4–5 d apart in the following order: control, RU486, deoxycorticosterone, corticosterone, and aldosterone.

Systolic BP (tail cuff method) and body weight were recorded weekly. After 8 wk of treatment, the rats were killed by decapitation, and the hearts were excised, blotted, and weighed. The heart was divided in the mid-coronal plane, and the apex was snap frozen and stored for biochemical analysis. The remaining heart tissue was immersion fixed in 4% buffered paraformaldehyde (pH 7.4) for histological processing and analysis.

During the experimental period, two rats receiving aldosterone died under anesthesia at the time of pump replacement, and one aldosterone rat and one DOC rat were killed at weeks 6 and 7 after their condition deteriorated.

Biochemical analysis. To estimate the collagen content of the heart, hydroxyproline (OHP) concentration was measured by a colorimetric assay as described by Chiariello et al. (9). Briefly, lyophylized heart tissue was digested overnight in 6 M HCl, and hydrolysates were diluted to a constant volume with further acid. Aliquots of 200 μl were vacuum dried at 110°C and were subsequently made back up to volume with distilled water. Oxidation of the samples with chloramine-T results in the formation of a pyrrole that in turn forms a colored compound when reacted with Erlich's reagent. OHP content of the samples was expressed as milligrams of OHP per gram of dry tissue, with samples assayed in triplicate.

Morphological analysis. Fixed blocks of tissue were embedded in paraffin, and 3-μm-thick sections were cut from each block. The sections were deparaffinized, rehydrated, and then placed in 0.2% phosphomolybdic acid (Univar, Sydney, Australia) for 2 min to reduce nonspecific staining (10). Sections were then stained with 0.1% Sirius red (Polysciences Inc., Washington, DC) in saturated aqueous picric acid for 1 h, differentiated in 0.01% HCl for 30 s, and rapidly dehydrated and mounted in Depex (BDH Chemicals, Port Fairy, Australia).

Collagen volume fraction was determined by measuring the area of stained tissue within a given field and expressing that area as a proportion of the total area under observation. One section from each heart was analyzed at an objective magnification of 16 under green filtered light. Images gathered with a CCD video camera were digitized, and reference dark- and light-fields, respectively, were subtracted and divided from the image to correct for uneven background illumination. A grey level threshold was set for stained collagen, and the area stained was calculated as a percentage of the total area (BioScan, Inc., Edmonds, WA).

The sections were sampled in a nonbiased (blinded) systematic fashion (11). For analysis of left and right ventricle interstitial collagen, each ventricle was scanned in entirety on the microscope, and every fifth field was used for analysis. Fields containing vessels or artifacts were not included. In the left ventricle, 15–16 fields were analyzed for interstitial collagen, and in the right ventricle 9–10 fields were analyzed. A similar method was used to sample perivascular collagen, although in this case only those fields containing vessels were considered, so that a further 2–6 fields were analyzed for each heart. The change in perivascular collagen content between groups was estimated by measuring the total collagen present in sampled fields (i.e., fields containing at least one vessel) and correcting for interstitial collagen, as follows:

for each field: % area PVC = Total % area of collagen

− % area IC (% area IC = nonvascular area × mean % area IC);

where PVC is perivascular collagen, IC is interstitial collagen, nonvascular area is all regions of the field in question that do not contain any part of a vessel or its surrounding collagen network, and mean % area IC is the mean interstitial collagen volume fraction calculated for that heart. To examine the repeatability of measurement, after initial measurements in random order, the control samples were reanalyzed. Readings of mean % area IC (mean±SD) in the right ventricle were 1.38 (±0.17) and 1.20 (±0.19) on repeat analysis; in the left ventricle the equivalent values were 1.40 (±0.16) and 1.18 (±0.16).

To determine the degree of hypertrophy present in each ventricle, cross-sectional areas were measured from micrographs taken from the mid-coronal plane at a magnification of 15. Sections were traced directly on a calibrated digitizing tablet and calculated directly (Measure Software, Capricorn Scientific, Woori Yallock, Victoria, Australia). Sections analyzed were stained with picrosirius red as described above, but at double the normal concentration to increase staining of noncollagen elements.

Statistical analysis. One-way ANOVA was performed on all data sets. Pearson's *r* value was determined for possible correlation between interstitial collagen levels in the left and right ventricles, for perivascular collagen levels, for OHP values, and for histologically determined collagen content.

The Duncan test for multiple comparisons was applied to all data sets in which multiple comparisons were made to test for statistical significance (12).

Results

Blood pressure, body weight, and cardiac hypertrophy. Administration of aldosterone or deoxycorticosterone for 8 wk increased systolic BP above control (Fig. 1, *top*) and was accompanied by a reduction in the rate of growth (Table I). RU486-treated rats showed a lower weight gain than control but no increase in blood pressure; corticosterone did not change either parameter. Similarly, the rats receiving aldosterone or deoxycorticosterone were the only groups to show an increase in both heart wet weight (Table I) and in the ratio of heart weight to body weight (Fig. 1, *bottom*). For both mineralocorticoids, the increase was highly significant ($P < 0.01$) and in the order of 40–50%.

Left ventricular hypertrophy, determined by measurement of cross-sectional area, was evident only in animals receiving aldosterone or deoxycorticosterone ($P < 0.01$ vs all groups)
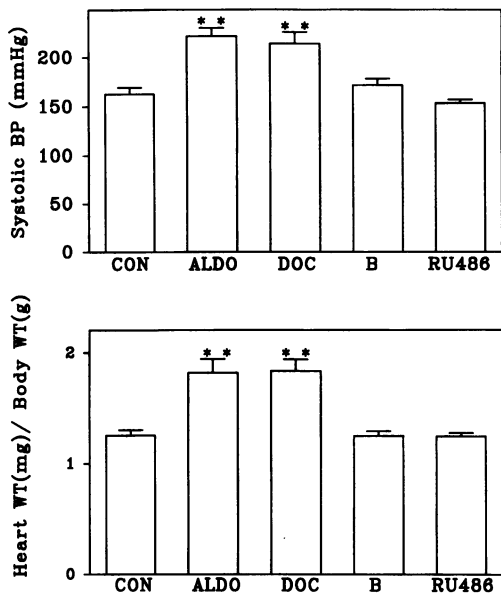
Figure 1. (*Top*) Systolic BP at the conclusion of the 8-wk period. (*Bottom*) Heart weight (mg)/body weight (g) as an index of cardiac hypertrophy. *CON,* control; *ALDO,* aldosterone; *B,* corticosterone. Values shown are mean±SEM; $n = 10$ except for *ALDO* ($n = 7$) and *DOC* ($n = 9$). ** $P < 0.01$ vs control.

(Fig. 2, *top*). In contrast, right ventricular wall cross-sectional area was unchanged in all experimental groups (Fig. 2, *bottom*).

Determination of OHP levels. As shown in Fig. 3, animals receiving aldosterone had cardiac OHP concentrations raised above all other groups, control and corticosterone ($P < 0.01$), deoxycorticosterone and RU486 ($P < 0.05$). Both the two latter groups also show a significant increase in levels above control ($P < 0.01$). Values for animals receiving corticosterone were not different from control.
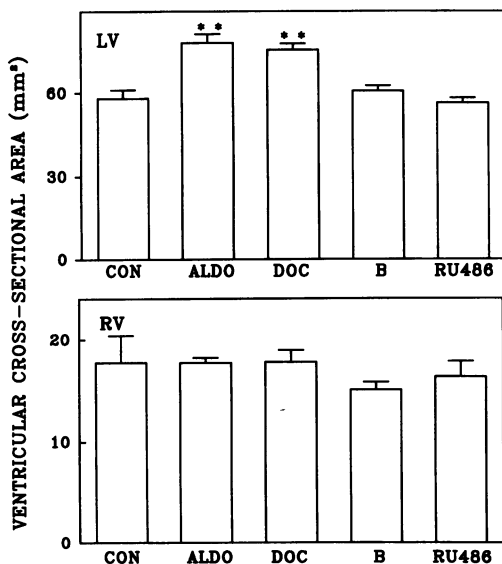

Figure 2. Left (*top*) and right (*bottom*) ventricular cross-sectional area (mm²). The left ventricle was taken to include the septum. *CON,* control; *ALDO,* aldosterone; *B,* corticosterone. For measurements of the right ventricular wall area $n = 7$ for control, 6 for *ALDO,* 9 for *DOC,* 7 for *B,* and 5 for *RU486.* ** $P < 0.01$ vs control.

Determination of interstitial collagen. As shown in Fig. 4, aldosterone produced the most marked increase in interstitial collagen volume fraction, with levels clearly higher than all other groups ($P < 0.01$). For aldosterone, collagen volume fraction was $\sim$ 2.5 times greater than control in both ventricles; values for all other groups were significantly raised above control ($P < 0.01$). Pearson's r value determined for comparison of interstitial collagen of the left and right ventricles is 0.94, indicating that they show an identical response ($P < 0.01$).

Determination of perivascular collagen. Fig. 5 shows that deoxycorticosterone administration is followed by a marked increase in perivascular collagen volume fraction (2.1-fold vs control, $P < 0.01$ vs all groups). Perivascular collagen was also found to be elevated in the RU486 group (1.4-fold vs control, $P < 0.05$ vs control), but not in groups receiving aldosterone or corticosterone. When control and aldosterone groups were compared post-hoc by t test, the aldosterone group was shown to be significantly higher ($P < 0.01$); in contrast, the corticosterone group was not significantly lower ($0.05 < P < 0.06$) than control.

Discussion

The findings of this study confirm those of Brilla and Weber (6), who showed the effects of mineralocorticoid administration on BP elevation and cardiac fibrosis. In addition, by documenting the effects of glucocorticoid agonist/antagonist administration on the same indices, this study has extended Brilla and Weber's work and suggests a plausible explanation for the clear difference between aldosterone and deoxycorticosterone effects noted in both series of studies. For reasons of practicality and economy, single dose regimens were used for all steroids; the amount of steroid and the frequency and route of administration were chosen on the basis of past experience as representing near–maximally effective dosage regimens.

In this study three different patterns of steroid response are seen. In terms of BP/cardiac hypertrophy, the classical mineralocorticoids are potent effectors, with neither the glucocorticoid or antiglucocorticoid producing a change over control. In contrast with the left ventricle, right ventricular cross-sectional area did not differ between groups. The variance in some groups is relatively high, reflecting a lower number of sections able to be scored than for left ventricle, in turn reflecting the incidence of tears, folds, etc. in some sections; nonetheless, there is no evidence for any increase in right ventricular wall thickness in mineralocorticoid-treated rats. This suggests that pressure in the pulmonary circulation is not elevated and, thus, that increased pressure is not required for the increased fibrosis seen with mineralocorticoid administration in the right, and by extrapolation the left, ventricle. Weber and his colleagues have presented data previously on the protective effect of subdepressor doses of spironolactone on aldosterone-induced cardiac interstitial fibrosis (7) and have similarly interpreted their findings as evidence for a humoral, rather than mechanical, etiology of the fibrotic response.

In terms of interstitial cardiac collagen fraction, although all steroids appear to elevate collagen to some extent, aldosterone is markedly and highly significantly more potent than any other treatment, including deoxycorticosterone. For perivascular collagen the converse is true, with deoxycorticosterone clearly the most potent effector; moreover, the antiglucocorticoid RU486 also produces significant perivascular fibrosis,

Table I. Body Weight and Heart Weight: Effects of Steroids

Group	n	Start weight	End weight	Δ Weight	Heart weight
		g	g	g	mg
Control	10	186±2	386±7	201±24	486±21†
Aldosterone	7	194±5	312±9*	118±27*	565±33†
Deoxycorticosterone	9	181±4	338±9*	158±24*	615±28*
Corticosterone	10	182±4	367±4	185±14	460±16
RU486	10	193±5	350±10*	158±24*	436±13

End weight is rat body weight at time of killing. Heart weight is the wet (blotted) weight of the heart, trimmed of major vessels. Values shown are mean±SEM. *$P < 0.01$ vs control. †$P < 0.05$ vs control.

whereas aldosterone does not. In addition, there is clearly a tendency for perivascular collagen to be lower in the group receiving corticosterone than in controls, with levels significantly lower than in the other three steroid-treated groups. The challenge, then, is to accommodate these findings (and those of Brilla and Weber [6], which were very similar for aldosterone and deoxycorticosterone) into our current understanding of mineralocorticoid action and then to discuss the extent to which the collagen responses found mirror physiologic or pathophysiologic events.

In terms of BP, the distinction between aldosterone and deoxycorticosterone on the one hand and corticosterone/RU486 on the other was not unexpected. From the work of Gomez-Sanchez and colleagues (13–15), the hypertensinogenic effect of aldosterone is crucially dependent on agonist occupancy of circumventricular type I (mineralocorticoid) receptors. Intracerebroventricular infusion of aldosterone, at doses without discernible effect when given systemically, progressively raised BP in uninephrectomized rats given 1% NaCl solution to drink. This effect was not mimicked by infusion of the specific type II (glucocorticoid) receptor agonist RU26988 nor by corticosterone (14); on the other hand, intracerebroventricular infusion of corticosterone, at doses similar to those of aldosterone, blocked the effect of intracerebroventricular aldosterone (14). More recently, Gomez-Sanchez et al. (15) have shown that intracerebroventricular infusion of RU28318, a highly specific mineralocorticoid receptor antagonist, will block the hypertensinogenic effect of peripherally infused aldosterone, at doses of RU28318 that do not affect the peripheral effects of the administered aldosterone, and which when given

peripherally do not block the hypertensinogenic effect of aldosterone.

Taken together, these data very strongly support the hypothesis that in the circumventricular organs aldosterone (and presumably deoxycorticosterone) acts via type I receptors that are unprotected by the enzyme 11βOH steroid dehydrogenase, in contrast with those in kidney and other epithelia. Secondly, the receptors involved also clearly differ in another way from those in epithelial tissue, for which there is excellent evidence that physiologic glucocorticoids are agonists when they occupy mineralocorticoid receptors (16).

For interstitial collagen volume deposition, the hierarchy of effects is different in several ways. The finding that all steroids used elevate interstitial collagen to some degree may reflect the more or less increased peripheral type I receptor occupancy that will occur in all four treatment groups. What remains unexplained is why aldosterone appears much more potent than deoxycorticosterone if the effect is mediated via type I mineralocorticoid receptors. One possible explanation would be that the dose of deoxycorticosterone is submaximal. This is possible, in the absence of dose–response curves, but highly unlikely
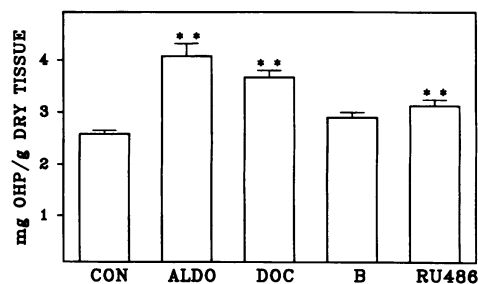

Figure 3. Biochemical determination of hydroxyproline concentration in the heart (*mg OHP/g tissue dry weight*). *CON,* control; *ALDO,* aldosterone; *B,* corticosterone. Values are mean±SEM; n = 10 except for *ALDO* (n = 7) and *DOC* (n = 9). **$P < 0.01$ vs control.

Figure 4. Interstitial collagen volume fraction for left (*top*) and right (*bottom*) ventricles (percentage of total area). *CON,* control; *ALDO,* aldosterone; *B,* corticosterone. Values are mean±SEM; n = 10 except for *ALDO* (n = 7) and *DOC* (n = 9). **$P < 0.01$ vs control.

Figure 5. Index of perivascular collagen (arbitrary units). CON, control; ALDO, aldosterone; B, corticosterone. Values are mean±SEM; $n = 10$ except for ALDO ($n = 7$) and DOC ($n = 9$). **$P < 0.01$ vs control.
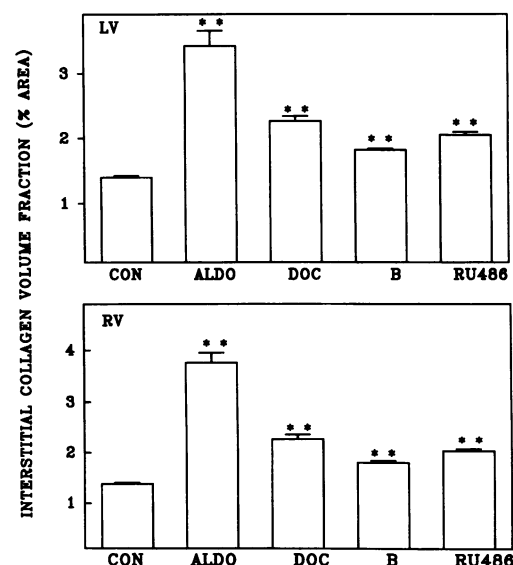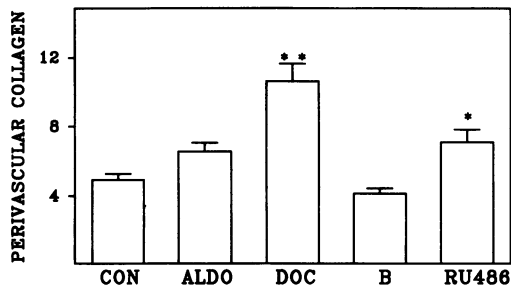
given the similar effects on BP and the much greater effect of deoxycorticosterone on perivascular fibrosis. A second possibility is the operation of a gatekeeper enzyme, in the heart and/or in epithelial tissues, other than 11βOH steroid dehydrogenase. It has been suggested recently (17) that 3α,20βOH steroid dehydrogenase may be such an enzyme, to exclude deoxycorticosterone and progesterone from type I receptors, but not aldosterone, which does not appear to be a substrate (18). Indirect evidence supporting such an action in excluding deoxycorticosterone has come from immunocytochemical studies in the kidney (19); its importance in epithelial tissues, or in the context of the interstitial cardiac fibroblast, has yet to be addressed formally.

In terms of perivascular collagen deposition, the most plausible explanation is that such deposition reflects the net effect of agonist occupancy of type I receptors and antagonist occupancy of type II receptors. By this criterion, deoxycorticosterone would be predicted to have the greatest effect, in that it is both a potent mineralocorticoid receptor agonist and a glucocorticoid receptor antagonist (20). RU486, which also significantly raises perivascular collagen, is a glucocorticoid receptor antagonist that will produce net elevation of corticosterone given in vivo and thus fractionally higher type I receptor occupancy by corticosterone, particularly if the perivascular type I receptors are unprotected. Aldosterone is a mineralocorticoid agonist but at higher doses is also a glucocorticoid agonist. Although the infused doses probably have little if any glucocorticoid agonist activity, there is clearly no type II receptor antagonism when aldosterone is infused.

The clear difference in response patterns between interstitial and perivascular collagen deposition makes it highly likely that at least one, and very possibly both, of these events reflects direct actions of mineralocorticoids and glucocorticoids on cardiac fibroblasts. The heart has been shown to contain type I receptors by in vitro ligand-binding studies (21) and relatively high levels of mRNA for such receptors (2). In vivo, tritiated corticosterone injected into adrenalectomized rats is excluded from type I receptors in kidney, colon, and parotid, but not in hippocampus and heart (4). Though 11βOH steroid dehydrogenase activity has been shown in both hippocampus (22) and heart (23), it reflects the NADP-preferring, lower affinity species (termed 11βHSD1), clearly distinct from the high affinity, NAD-preferring species shown to be responsible for excluding glucocorticoids from type I receptors in physiological aldosterone target tissues (24). Given the presumed physiological role of 11βHSD1 in modulating glucocorticoid receptor occupancy

by physiological glucocorticoids, it may well be that its presence in the heart may have more relevance to perivascular fibrosis, in which glucocorticoid receptor–mediated effects are likely, than to interstitial fibrosis.

The measured levels of perivascular collagen after 8 wk of steroid exposure presumably reflect the result of increased collagen synthesis and/or decreased collagen breakdown. Increased collagen synthesis, in vivo and that reported for fibroblasts in vitro (8), may reflect agonist effects of aldosterone or deoxycorticosterone via mineralocorticoid receptors. If this is the case, one possible explanation for the marked perivascular effect of glucocorticoid antagonists (RU486, deoxycorticosterone) may be that they decrease collagenase activity; implicit in such an explanation is that in the longer term glucocorticoids have a tonic effect on collagenase gene expression. Such a possibility is consistent with the studies of Jonat et al. (25), who showed that in addition to its effect on downregulating collagenase gene expression via an interaction of the activated glucocorticoid receptor with AP-1, dexamethasone may increase collagenase expression in the absence of AP-1 activation. One interpretation of these molecular events is that at high levels of inflammatory mediators, such as are present in acute inflammatory responses, glucocorticoids may decrease collagenase gene expression and thus may be antiinflammatory; when mediator levels fall, glucocorticoids may increase collagenase levels, against chronic or reactive fibrotic responses.

In conclusion, the physiologic, pathophysiologic, and therapeutic implications of these findings deserve brief discussion. Brilla and Weber (6) have shown that equivalent aldosterone infusion on a low salt intake results in control levels of cardiac fibrosis (and BP) and interpret these findings as evidence for the importance of an aldosterone-salt imbalance, though how the salt effect is mediated remains unclear. From our data, it would appear that interstitial collagen deposition is relatively easily elevated, even by modest changes in type I receptor occupancy, and is independent of BP elevation. In terms of therapeutic implications, deoxycorticosterone is very rarely used, and if the mineralocorticoid agonist/glucocorticoid antagonist activity of DOC explains its very marked effect on perivascular collagen deposition, 9α-fluorocortisone would clearly be a much less active steroid in this regard, as it is both a potent mineralocorticoid and glucocorticoid agonist. Finally, though currently RU486 is being largely used on a once-off basis as a contragestive, it is also being used as a progesterone antagonist in patients with meningioma, and it or its derivatives may become widely used on a chronic basis for fertility regulation. Where administration is on a chronic basis, cardiac perivascular fibrosis and its consequences may thus need to be borne in mind as possible side effects.

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