Aldosterone Synthase Inhibitor Ameliorates Angiotensin II–Induced Organ Damage

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Background—Aldosterone and angiotensin (Ang) II both may cause organ damage. Circulating aldosterone is produced in the adrenals; however, local cardiac synthesis has been reported. Aldosterone concentrations depend on the activity of aldosterone synthase (CYP11B2). We tested the hypothesis that reducing aldosterone by inhibiting CYP11B2 or by adrenalectomy (ADX) may ameliorate organ damage. Furthermore, we investigated how much local cardiac aldosterone originates from the adrenal gland.

Methods and Results—We investigated the effect of the CYP11B2 inhibitor FAD286, losartan, and the consequences of ADX in transgenic rats overexpressing both the human renin and angiotensinogen genes (dTGR). dTGR-ADX received dexamethasone and 1% salt. Dexamethasone-treated dTGR-salt served as a control group in the ADX protocol. Untreated dTGR developed hypertension and cardiac and renal damage and had a 40% mortality rate (5/13) at 7 weeks. FAD286 reduced mortality to 10% (1/10) and ameliorated cardiac hypertrophy, albuminuria, cell infiltration, and matrix deposition in the heart and kidney. FAD286 had no effect on blood pressure at weeks 5 and 6 but slightly reduced blood pressure at week 7 (177±6 mm Hg in dTGR+FAD286 and 200±5 mm Hg in dTGR). Losartan normalized blood pressure during the entire study. Circulating and cardiac aldosterone levels were reduced in FAD286 or losartan-treated dTGR. ADX combined with dexamethasone and salt treatment decreased circulating and cardiac aldosterone to barely detectable levels. At week 7, ADX-dTGR-dexamethasone-salt had a 22% mortality rate compared with 73% in dTGR-dexamethasone-salt. Both groups were similarly hypertensive (190±9 and 187±4 mm Hg). In contrast, cardiac hypertrophy index, albuminuria, cell infiltration, and matrix deposition were significantly reduced after ADX (P<0.05).

Conclusions—Aldosterone plays a key role in the pathogenesis of Ang II–induced organ damage. Both FAD286 and ADX reduced circulating and cardiac aldosterone levels. The present results show that aldosterone produced in the adrenals is the main source of cardiac aldosterone. (Circulation. 2005;111:3087-3094.)

Key Words: aldosterone ▪ angiotensin ▪ heart failure ▪ renin ▪ inflammation

Since the publication of 2 clinical trials, RALES (Randomized Aldactone Evaluation Study) and EPHECUS (Eplerone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study), the role of aldosterone in cardiovascular remodeling has generated considerable attention.1,2 The key enzyme in aldosterone production is aldosterone synthase (CYP11B2). CYP11B2 is predominantly expressed in the adrenal gland but is also expressed in the cardiovascular system.3 Angiotensin (Ang) II is the main stimulus for CYP11B2-related aldosterone synthesis. Preclinical and clinical studies have shown that Ang II inhibition is pivotal to the treatment of heart failure and ischemic heart disease. The previous belief was that inhibition of Ang II should be sufficient to block aldosterone production; however, aldosterone levels can be elevated even though Ang II production is inhibited or its action is blocked.4 This state of affairs is called the aldosterone breakthrough phenomenon; its mechanisms are unclear. Blocking the mineralocorticoid receptor (MR) reduces proteinuria in ACE inhibitor–treated patients with early diabetic nephropathy.5 MR is directly involved in ischemia-induced cardiac remodeling. Hayashi et al6 prevented postinfarct left ventricular remodeling with spironolactone added to an ACE inhibitor. However, whether aldosterone in the heart tissue is derived from the circulating blood or is produced locally, thereby contributing to remodeling, is unclear. We investigated FAD286, a novel CYP11B2 inhibitor...
inhibitor, in an Ang II–dependent rat model of organ damage. The rats are transgenic for the human renin and angiotensinogen genes (dTGR). The animals develop hypertension and severe cardiac and renal damage. They die between 7 and 8 weeks of age.7–10 In previous studies, we demonstrated that MR blockade with spironolactone or eplerenone prevented mortality and Ang II–induced organ damage.9,10 The aim of the present study was to define the effect of FAD286 and the role of circulating or locally produced aldosterone in the pathogenesis of Ang II–induced renal and cardiac damage.

Methods

In Vitro Analysis of FAD286 in NCI-H295R Cells

Human adrenocortical carcinoma NCI-H295R cells (American Type Collection, Manassas, Va) were seeded in NBS 96-well plates at a density of 25 000 cells/well in 100 μL of a growth medium containing DMEM/F12 (Gibco) supplemented with 10% FCS (Gibco), 2.5% Nu-serum (BD Biosciences), 1 μg TFS/mL (insulin/transferrin/seleumin; Gibco), and 1x antibiotic/antimycotic (Gibco). The medium was changed after being cultured for 3 days at 37°C under an atmosphere of 5% CO2/95% air. On the following day, cells were rinsed with 100 μL of DMEM/F12 and incubated with 100 μL of treatment medium containing 1 μmol/L Ang II (Sigma) and FAD286 at different concentrations in quadruplicate wells at 37°C for 24 hours. Immunoreactive aldosterone (ir-Ald) was measured in the supernatant by radioimmunoassay developed by Novartis Ltd.

Animal Studies

Experiments were conducted in 4-week-old male dTGR.11 The rats were kept in rooms at 24 ± 2°C and fed a standard rat diet containing sodium at 2 g/kg. They had free access to tap water or 1% saltwater depending on the protocols. All American Physiological Society guidelines for animal care were followed (permit No. G 408/97). Three different treatment protocols were performed. Systolic blood pressure was measured weekly by tail cuff. Urine was collected over 24 hours. Urinary albumin was measured by ELISA (CellTrend). Three different treatment protocols were performed. Systolic blood pressure was measured weekly by tail cuff. Urine was collected over 24 hours. Urinary albumin was measured by ELISA (CellTrend). Rats were killed at 7 or 9 weeks of age, and kidneys and hearts were excised, washed with ice-cold saline, blotted dry, weighed, and stored at −80°C. Serum was collected and stored at −20°C. Plasma renin concentration (PRC) and corticosterone and serum and cardiac ir-Ald levels were determined according to previously published methods.12,13 Serum corticosterone was extracted from plasma with organic solvents and measured by specific radioimmunoassay wit

Immunohistochemistry

Ice-cold acetone-fixed cryosections (6 μm) of renal and cardiac tissue were stained by the alkaline phosphatase/anti–alkaline phosphatase technique and immunofluorescence as described previously.7 To determine cell infiltration, sections were incubated with the following monoclonal antibodies: anti-CD4 (Pharmingen), anti-ED-1, anti-CD8 (Serotec), anti-major histocompatibility complex class II, anti-CD86, and anti-Ox6 (all BD Pharmingen). Polyclonal antibodies for anti-fibronectin (Paesel) and anti-collagen IV (Southern Biotechnology) were used to visualize fibrosis. Semiquantitative scoring of infiltrated cells, in 15 different cortical kidney and cardiac areas (n=5 per group), was done with samples examined in a blinded fashion. Collagen IV and fibronectin expression were presented in arbitrary units (0 to 5 +) based on staining intensity.

RNA Expression With TaqMan

For reverse transcription–polymerase chain reaction (RT-PCR), RNA was isolated from left ventricular tissue according to the TRIZOL protocol (Gibco Life Technology), and cDNA was transcribed with superscript II according to the protocol. Primers (185, hypoxanthine phosphoribosyl transferase gene, α- and β-myosin heavy chain [MHC], atrial natriuretic peptide, MR, 11β-hydroxysteroid dehydrogenase, and CYP11B2; primer sequences available on request) were synthesized by Biotec. For CYP11B2, MR, and 11β-hydroxysteroid dehydrogenase, RT-PCR was performed with the TaqMan system (Prism 7700 Sequence Detection System, PE Biosystems). Forty-five cycles of PCR were performed according to the PCR TaqMan-Mastermix (Applied Biosystems) protocol instructions. For α-MHC, β-MHC, and atrial natriuretic peptide, quantitative RT-PCR amplification was performed in 25 μL of SYBRGreen PCR Master Mix (Applied Biosystems) containing 0.3 or 0.9 mol/L primer and 1 μL of the reverse-transcription reaction with a 5700 Sequence Detection System (Applied Biosystems). Thermal cycling conditions comprised an initial denaturation step at 95°C for 10 minutes, followed by 95°C for 15 seconds and 65°C for 1 minute for 40 cycles. mRNA expression was normalized relative to the housekeeping genes 18S and hypoxanthine phosphoribosyl transferase.

Statistical Analysis

Data are generally expressed as mean±SEM. Plasma renin serum and cardiac aldosterone concentrations are presented as median value (range). Statistically significant differences in mean values were tested by ANOVA and in blood pressure by repeated-measures ANOVA, followed by the Scheffé test as indicated. Nonparametric testing was performed for differences between median values. Mortality is presented as Kaplan-Meier analysis. A value of P<0.05 was considered statistically significant. The data were analyzed with Statview statistical software.

Results

FAD286 and Its Effect on Ang II–Induced Aldosterone Production in NCI-H295R Cells

FAD286 [(+)-(5R)-4-(5,6,7,8-tetrahydroimidazo [1,5-alpyridin-5-yl] benzonitrile hydrochloride] is shown in Figure 1A. FAD286 dose dependently inhibited the Ang II–induced generation of ir-Ald in NCI-H295R cells with an IC50 of 37 nmol/L (Figure 1B).

Effect of FAD286 and Losartan on Organ Damage

At week 7, untreated dTGR showed 40% mortality (5/13), whereas only 1 of 10 FAD286-treated and no losartan-treated dTGR died before the end of the study at week 7 (Figure 2A). Systolic blood pressure in untreated dTGR increased progressively from week 5 to week 7. FAD286-treated dTGR also showed elevated blood pressure at weeks 5 and 6 that was slightly reduced at week 7 compared with untreated dTGR (200±5 and 177±6 mm Hg, P<0.05; Figure...
2B). In contrast, losartan normalized blood pressure (107±5 mm Hg, P<0.05; Figure 2B).

FAD286 and losartan both significantly reduced the cardiac hypertrophy index compared with untreated dTGR (4.4±0.1, 3.1±0.03, and 4.9±0.2 mg/g, respectively, P<0.05; Figure 2C). Echocardiographic analysis demonstrated that untreated dTGR developed concentric cardiac hypertrophy that was reduced by FAD286 and normalized by losartan. Total wall thickness (expressed as the sum of the septum plus left ventricular posterior wall) of dTGR was 3.7±0.01 mm with a normal left ventricular end-diastolic diameter. FAD286 and losartan significantly reduced wall thickness to 3.3±0.004 and 2.5±0.006 mm, respectively, P<0.05 (Figure 2D). Atrial natriuretic peptide mRNA was increased in untreated dTGR compared with both treatment groups (Figure 2E). Furthermore, untreated dTGR showed an α-MHC shift to the fetal β-MHC phenotype, which indicates cardiac hypertrophy. The MHC shift to the fetal β-isof orm was slightly reduced by FAD286 and completely reversed by losartan (Figure 2E).

Aldosterone synthase and Ang II type 1 receptor inhibition improved renal function. Urinary albumin excretion at week 7 was significantly higher in untreated dTGR than in FAD286-treated or losartan-treated dTGR (20.3±4.3, 9.6±1.7, and 0.6±0.14 mg/d, respectively, P<0.05; Figure 3A). Chronic FAD286 or losartan treatment reduced the infiltration of macrophages (Figure 3B), CD4 (Figure 3C) and CD8 T-cells, dendritic cells, major histocompatibility complex class II–positive cells, and CD86-positive cells (data not shown) in the kidney to a similar extent. FAD286 and losartan also reduced collagen IV (Figure 3D) and fibronectin (data not shown) deposition in the kidney. Fibronectin and collagen IV semiquantification demonstrated that losartan was slightly more effective (a score of 5+ for dTGR, 2+ for dTGR+FAD286, and 1+ for dTGR+losartan).

**Effect of ADX on Organ Damage**

To elucidate the role of aldosterone in the pathogenesis of Ang II–induced organ damage in more detail, we adrenalectomized dTGR at 4 weeks of age. The cumulative analysis from protocols II and III showed a 73% mortality rate of dTGR-dexamethasone-salt rats at week 7, whereas only 22% of ADX-dTGR-dexamethasone-salt rats died (Figure 4A). In protocol III at week 9, all dTGR-dexamethasone-salt rats were dead, whereas only 38% of ADX-dTGR-dexamethasone-salt rats had died (data not shown). ADX had no effect on systolic blood pressure (190±9 and 187±4 mm Hg at week 6, respectively; Figure 4B). Cardiac hypertrophy index was significantly more elevated in dTGR-dexamethasone-salt rats than in ADX-dTGR-dexamethasone-salt rats (5.7±0.4 and 4.5±0.1 mg/g, P<0.05; Figure 4C). Macrophage infiltration in hearts of ADX rats was significantly reduced compared with dTGR-dexamethasone-salt rats (not shown). At week 6, albuminuria was reduced in ADX-dTGR-dexamethasone-salt rats to almost normal levels, namely, 1.5±0.7 mg/d compared with 47.8±18.3 mg/d in dTGR-dexamethasone-salt rats (P<0.05; Figure 4D). Deposition of collagen IV in the kidney (a score of 4+ for dTGR-dexamethasone-salt and 1+ for ADX-dTGR-dexamethasone-salt; Figure 4E) and fibronectin in the heart (a score of 4+ for dTGR-dexamethasone-salt and 2+ for ADX-dTGR-dexamethasone-salt; Figure 4F) was significantly reduced after ADX. Renal CD4, CD8 T-cell, and macrophage infiltration, as well as dendritic cell and CD86-positive cell infiltration, were all significantly reduced in ADX rats (data not shown). These data document that ADX markedly improves renal and cardiac damage in

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**Figure 1.** A, FAD286 structure. B, FAD286 dose dependently inhibits Ang II–stimulated ir-Ald production in NCI-H295R human adrenocortical carcinoma cells.

(+)-(5R)-4-(5,6,7,8-tetrahydroimidazo[1,5-a]pyridin-5-yl) benzonitrile hydrochloride

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Aldosterone synthase and Adrenalectomy, and Ang II

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salt-treated dTGR. In protocol III, the rats were followed up to week 9. FAD286 treatment of ADX-dTGR-dexamethasone-salt rats reduced mortality to 28% without affecting blood pressure. The treatment significantly reduced infiltration of macrophages and T cells in the heart (data not shown).

Effect of FAD286 on Plasma and Cardiac Aldosterone, Plasma Renin, and Corticosterone FAD286 and losartan treatment both reduced circulating and cardiac levels of ir-Ald compared with untreated dTGR. Median values (ranges) were as follows: serum aldosterone 136 (81–454), 131 (8–296), and 408 (114–1410) pg/mL,
respectively, and cardiac aldosterone 458 (274–827), 500 (173–3460), and 3826 (302–14286) pg/g wet weight, respectively (Figure 5A). The highest cardiac and plasma aldosterone levels were found in untreated dTGR with the most severe organ damage. Cardiac and plasma aldosterone concentrations were reduced in parallel by the treatments. Untreated dTGR showed very high total PRC with a median value at 53 (13–173) ng · mL⁻¹ · h⁻¹. The highest PRC was again observed in untreated dTGR with the most severe organ damage. FAD286 treatment reduced serum aldosterone by 67%, from 408 to 136 pg/mL. Losartan treatment had an effect similar to that of FAD286 on serum aldosterone levels. FAD286 and losartan treatment did not affect PRC or serum corticosterone levels. Median PRC levels for FAD286- and losartan-treated dTGR were 8.0 (range 5.3 to 50) and 41 (range 13 to 57) ng · mL⁻¹ · h⁻¹; corticosterone levels were 459 (range 103 to 524), 416 (range 187 to 563), and 395 (range 310 to 409) ng/mL, respectively.

Effect of ADX on Plasma and Cardiac Aldosterone, Plasma Renin, and Corticosterone
Dexamethasone-salt treatment (Figure 5B) of dTGR resulted in an 85% suppression of PRC, from a median value of 53 (13–173) ng · mL⁻¹ · h⁻¹ in untreated dTGR to low normal 8.0 (8.0 to 48) ng · mL⁻¹ · h⁻¹ (normal PRC in Sprague-Dawley rats is 23 (5.3 to 36) ng · mL⁻¹ · h⁻¹). At the same time (week 7), dexamethasone-salt treatment reduced serum aldosterone by 84%, from 408 (114–1410) to 65 (25–699) pg/mL, and cardiac aldosterone by 95%, from a median of 3826 (302–14286) to 267 (102–3621) pg/g. Serum corticosterone decreased in a similar pattern, from 459 (103–524) ng/mL in untreated dTGR to 173 (57–317) ng/mL in dTGR-dexamethasone-salt rats (−62%). dTGR-dexamethasone-salt rats that underwent additional ADX showed a further reduction of serum and cardiac aldosterone to 2.0 (<1.6 to 10) pg/mL (−99.5%) and to 27 (13–45) pg/g (−99.3%), respectively, whereas PRC remained low at 16 (8–28) ng · mL⁻¹ · h⁻¹. In parallel to the aldosterone vanishing after ADX, serum corticosterone also disappeared (assay detection limit <1 ng/mL).

By week 9, cardiac aldosterone had fallen below 20 pg/g in all rats (10 [<6.7 to 19] pg/g). Additional treatment of ADX-dTGR-dexamethasone-salt rats with FAD286 resulted in undetectable serum aldosterone (<1.6 pg/mL) in 7 of 8 rats and cardiac aldosterone values below 10 pg/g in all 5 hearts tested.

Discussion
We found that aldosterone production inhibition by FAD286 or ADX protected rats from Ang II–induced inflammatory and fibrotic organ damage. The present data also demon-
strated that the main source of cardiac aldosterone in the dTGR model is the adrenal gland. These results show the first description of protection via aldosterone synthase inhibition in vivo. We found previously that MR blockade protects against Ang II–induced organ damage. The MR antagonists spironolactone and eplerenone also reduced mortality and ameliorated renal and cardiac damage in dTGR rats.9,10 Rocha et al15 showed that MR blockade prevents Ang II/salt-induced vascular inflammation in the rat heart. One explanation for this effect might be the interaction between the Ang II receptor and MR. Xiao et al16 demonstrated the aldosterone-potentiated, Ang II–induced proliferation of vascular smooth muscle cells. We showed that aldosterone potentiated Ang II–induced extracellular signal-regulated kinase-1/2 (ERK1/2)mitogen-activated protein kinase signaling in vascular smooth muscle cells. In addition, Ang II–induced ERK1/2 phosphorylation depends on a functioning MR.9 The effects are mediated in part via the generation of oxygen radicals. Keidar et al17 demonstrated that the aldosterone antagonist eplerenone reduced oxidative stress in serum and peritoneal macrophages of apolipoprotein E knockout mice, which correlated with a significant reduction in atherosclerotic lesion area. Taken together, these findings fit well with observations made by Schiffrin et al18 more than 20 years ago that demonstrated an interaction between the aldosterone and Ang II signaling pathways.

Besides the steroidogenic acute regulatory protein that moves cholesterol into the mitochondria (StAR), the key enzyme in the production of aldosterone is the aldosterone synthase or CYP11B2. CYP11B2 was isolated in 1992 by Kawamoto et al19 and was mapped to human chromosome 8q24.3.20 The enzyme is expressed in the adrenal gland zona glomerulosa and determines circulating aldosterone levels. CYP11B2 mRNA expression has also been found in blood vessels and brain.21,22 However, the physiological relevance of these findings is currently not clear. In the present animal model, depletion of circulating aldosterone after ADX and reduction of aldosterone due to pharmacological intervention with the aldosterone synthase inhibitor FAD286 and the Ang II type 1 receptor blocker losartan ameliorated Ang II–induced renal and cardiac damage. Chander et al23 showed similar results in saline-drinking, stroke-prone spontaneously hypertensive rats (SHRSP), in which ADX prevented the development of thrombotic microangiopathy. The authors suggested that aldosterone was the major pathogenic stimulus in this animal model, because only resubstitution with aldosterone, not Ang II, caused thrombotic microangiopathy in ADX-SHRSP.

Ang II and a high extracellular potassium concentration stimulate CYP11B2 activity. High salt suppresses circulating aldosterone levels.24 In the present model, dexamethasone-

Figure 4. A, ADX reduced mortality in dTGR. B, Systolic blood pressure at week 6 was not different between 2 groups. C, ADX reduced cardiac hypertrophy. D, Albuminuria was reduced in ADX rats. E, Renal collagen IV immunoreactivity was reduced after ADX. F, ADX-dTGR-dexamethasone-salt showed less cardiac fibrosis (fibronectin immunoreactivity) than dTGR-dexamethasone-salt. Results are mean±SEM (*P<0.05 vs dTGR-dexamethasone-salt).
salt treatment also led to 6-fold lower circulating aldosterone levels and 14-fold lower cardiac aldosterone levels. Takeda et al.21 observed a reduction in circulating aldosterone levels in sodium-loaded SHRSP; however, in contrast to the present results, they described an enhanced cardiac and vascular aldosterone synthesis in sodium-treated animals.21,25 Very recently, Gomez-Sanchez et al.26 showed that in healthy rats, cardiac aldosterone levels were determined by aldosterone production in the adrenal gland. The present data in transgenic ADX rats confirm their findings and extend them to a pathophysiological situation. Three weeks after ADX, ADX-dTGR-dexamethasone-salt rats showed only a very small amount of ir-Ald in the heart, which equaled only 10% of cardiac aldosterone in dTGR-dexamethasone-salt rats with intact adrenals and 0.7% in untreated dTGR. At 5 weeks after ADX, cardiac ir-Ald was 3-fold lower than 3 weeks after ADX. These data suggest that in the present transgenic model, >99% of cardiac aldosterone is not produced locally, and the major source of aldosterone originates from the adrenal gland. The high cardiac aldosterone levels and the high cardiac/plasma ratio in untreated dTGR surprised us. In nontransgenic Sprague-Dawley controls, plasma aldosterone was 143 (range 79 to 226) pg/mL, and cardiac aldosterone was 140 (range 80 to 654) pg/g. These values correspond to those reported by Gomez-Sanchez et al.26 However, when we compared these values with those found in the present dTGR model, we found only about a 3-fold increase in plasma levels but a 30-fold increase in cardiac levels. This suggests that substantial amounts of circulating aldosterone might be taken up into the heart. In support of this interpretation is the fact that we also found a strong correlation between plasma and cardiac aldosterone levels.

The role of locally produced aldosterone and its pathophysiological significance are currently debated.27,28 Silvestre et al.29 found increased aldosterone concentrations after Ang II or adrenocorticotropic hormone administration in isolated perfused hearts of Wistar rats. Aldosterone production by the failing heart has been suggested on the basis of catheter-obtained aldosterone concentrations across the heart.30 Studies of patients with acute myocardial infarction and with congestive heart failure have demonstrated that aldosterone is taken up from the circulation into the injured heart.31,32 However, in patients with hypertrophic cardiomyopathy, a 7-fold upregulation of cardiac CYP11B2 mRNA was reported.33 The latter finding suggests local production of aldosterone in addition to uptake of the hormone from the circulation. We also addressed the issue of CYP11B2 expression in failing hearts in the present model. We analyzed CYP11B2 mRNA expression in 2 dTGR subgroups, dTGR with cardiac hypertrophy and dTGR with terminal heart failure. Nevertheless, no CYP11B2 upregulation was observed (unpublished data). In addition, we found low levels of circulating and cardiac aldosterone at 3 weeks after ADX. These values decreased further at 5 weeks after ADX and fell partly below the detection limit. Thus, the present data suggest that in the dTGR model, most, if not all, cardiac aldosterone was generated in the adrenal glands, released into the bloodstream, and taken up by the heart. The amount of aldosterone necessary to regulate salt balance is 100 to 1000 times less than the cortisol concentration needed to influence carbohydrate metabolism. Therefore, even small changes in aldosterone concentration might change the fate of cells and tissues locally. This state of affairs has been shown in vitro, where nanomolar concentrations of aldosterone potentiated epidermal growth factor–induced early signaling in Chinese hamster ovary cells34 or Ang II–induced signaling in vascular smooth muscle cells.9,16

In conclusion, the present results document the potential therapeutic utility of the novel aldosterone synthase inhibitor FAD286 in an Ang II–dependent hypertensive animal model. Depletion of aldosterone due to ADX prevented organ damage, although we cannot completely rule out a modest contribution by corticosterone. The present data demonstrate that in this model, cardiac aldosterone levels depend on aldosterone synthe-

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**Figure 5.** A and B, Serum and cardiac aldosterone concentrations (log scale). FAD286 and ADX decreased serum and cardiac aldosterone concentrations.
sis in the adrenal glands. Whether or not a local aldosterone production contributes to other forms of heart disease (ischemia, for example) deserves further study.\(^5\)

Acknowledgments

The present studies were supported by grants-in-aid from the NGFN (Nationales Genom Netzwerk) and by Novartis Institutes for BioMedical Research USA. Dr Muller holds a Helmholz fellowship. The Deutsche Forschungsgemeinschaft (DFG) supported Drs Muller and Luft. We thank M. Köhler, G. N’diaye, M. Schmidt, A. Schiche, J. Meisel, A. Weller, F. Nicoud, I. Keller, S. Novelli, and J. Czycki for their excellent technical assistance.

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_Circulation._ 2005;111:3087-3094; originally published online June 6, 2005; doi: 10.1161/CIRCULATIONAHA.104.521625
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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