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Effect of Acute Aldosterone Administration on Gene Expression Profile in the Heart

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Aldosterone is known to have a number of direct adverse effects on the heart, including fibrosis and myocardial inflammation. However, genetic mechanisms of aldosterone action on the heart remain unclear. This paper describes an investigation of temporal changes in gene expression profile of the whole heart induced by acute administration of a physiologic dose of aldosterone in the mouse. mRNA levels of 34,000 known mouse genes were measured at eight time points after aldosterone administration using oligonucleotide microarrays and compared with those of the control animals who underwent a sham injection. A novel software tool (CAGED) designed for analysis of temporal microarray experiments using a Bayesian approach was used to identify genes differentially expressed between the aldosterone-injected and control group. CAGED analysis identified 12 genes as having significant differences in their temporal profiles between aldosterone-injected and control groups. All of these genes exhibited a decrease in expression level 1–3 h after aldosterone injection followed by a brief rebound and a return to baseline. These findings were validated by quantitative RT-PCR. The differentially expressed genes included phosphatases, regulators of steroid biosynthesis, inactivators of reactive oxygen species, and structural proteins. Several of these genes are known to functionally mediate biochemical phenomena previously observed to be triggered by aldosterone administration, such as phosphorylation of ERK1/2. These results provide the first description of cardiac genetic response to aldosterone and identify several potential mediators of known biochemical sequelae of aldosterone administration in the heart. (*Endocrinology* 147: 3183–3189, 2006)

THERE IS AN extensive body of clinical and experimental evidence that aldosterone has direct adverse effects on the heart. More than 50 yr ago, Selye (1) reported that exogenous administration of mineralocorticoids in presence of a high-salt diet resulted in vascular inflammatory lesions in the heart. Patients with primary hyperaldosteronism have been shown to have impaired left ventricular diastolic function and prolonged PQ interval, compared with matched patients with essential hypertension (2–4), suggesting excessive collagen deposition and cardiac fibrosis. Excision of the aldosterone-producing adenoma corrects these alterations (3), highlighting the etiologic role of mineralocorticoid excess. Plasma aldosterone levels correlate positively with the severity of cardiac damage (5, 6).

Animal studies have demonstrated that mineralocorticoid excess in conjunction with a high sodium intake results in myocardial inflammation and fibrosis (7–9). Damage involves both left and right ventricle as well as both atria and the adventitia of the pulmonary artery, underlining blood pressure-independent nature of the phenomenon (10). These results were confirmed in a transgenic model of intracardiac mineralocorticoid excess overexpressing 11β-hydroxysteroid dehydrogenase type 2 in cardiomyocytes leading to cardiac fibrosis and heart failure (11).

Adverse effects of aldosterone are abrogated by administration of a mineralocorticoid receptor antagonist in animal models (8, 12–14), suggesting that induction of cardiac inflammation and fibrosis by aldosterone is mediated by the mineralocorticoid receptor activation. In clinical studies, mineralocorticoid receptor antagonists have been demonstrated to decrease morbidity and mortality in normotensive patients with heart failure (15, 16).

The mechanism by which activation of mineralocorticoid receptor leads to cardiac inflammation and fibrosis is unclear. Mineralocorticoid receptor functions as a ligand-dependent transcription factor. However, immediate consequences of mineralocorticoid receptor activation on gene expression in the heart are poorly understood (17). Most reports focus on either a narrow group of genes or a single time point after aldosterone administration (18, 19). However, a single time point is unlikely to capture the dynamic nature of gene expression (20–22). In this study we have taken a different approach by determining the temporal profile of the effects of acute *in vivo* aldosterone administration on gene expression profile in the heart. Importantly, we also included at each time point a response to a placebo injection, therefore separating the effects of the experimental method from those of the aldosterone.

Materials and Methods

Experimental animals

All experimental procedures involving mice were in accordance with guidelines of the Institutional Animal Care and Use Committee at Harvard University.

Male C57BL/6 mice (Charles River, Wilmington, MA) were uninephrectomized at 11 wk of age and allowed 7 d recovery time. Starting

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at 12 wk of age, all animals were fed 3% NaCl diet (Purina Test Diet Salt Series based on Basal Diet 5755, Pharmaserv Inc., Framingham, MA) for 1 wk. Animals were allowed unlimited access to water.

At the age of 13 wk, mice were administered ip injection of 125 μl mouse serum as control or 250 ng (approximately 10 μg/kg) aldosterone (Acros Organics, Geel, Belgium) diluted in 250 nl ethanol and subsequently in 125 μl mouse serum. Five animals in each of the control and experimental groups were killed at the following intervals after the injection: 0 h (no injection), 0.5, 1, 2, 3, 4, 5, and 12 h.

All mice were killed by deep inhalatory anesthesia with isoflurane. Blood and the heart were collected from every animal.

Biochemical assays

Plasma aldosterone was measured using a RIA kit (Diagnostic Products Corp., Los Angeles, CA). Plasma corticosterone levels were measured using a competitive enzyme immunoassay kit (American Laboratory Products Co., Windham, NH).

Microarray analysis

Mouse hearts were preserved in liquid nitrogen immediately after death of the animal. The total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA concentration and purity were assessed using a Beckman Coulter spectrophotometer DV 600. All RNA samples used in these experiments had 260:280 ratios between 1.94 and 2.25. Aliquots of the extracted total RNA were assessed for integrity by agarose gel electrophoresis and ethidium bromide staining for visualization of the 28S and 18S rRNA bands. RNA samples from all five animals receiving the same treatment at each time point were pooled.

Microarray analysis was performed using the mouse 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA), which contain probes for more than 34,000 known mouse genes. Preparation of the labeled cDNA and microarray hybridization were performed by the Microarray Core Facility at the Children's Hospital (Boston, MA). The raw data were normalized and analyzed using GeneChip Affymetrix GCOS 1.2 software. The signal values and the present, absent, or marginal calls were computed for all probe sets. Only probe sets with the average signal intensity greater than 1280 that were either unique for the gene or directed against the 3′ region of the gene were used in the analysis as described in detail in *Results*.

The ratios of signal intensities of aldosterone injection to control groups from each of the eight time points were used as an input for Cluster Analysis of Gene Expression Dynamics (CAGED) 1.0 (http://genomethods.org/caged), a program specifically designed for cluster analysis of temporal microarray experiments (23).

The data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE3440) (24, 25).

Quantitative real-time RT-PCR

PCRs were performed using total RNA generated for microarray analysis. cDNA was synthesized from 5 μg RNA with the first-strand cDNA synthesis kit (Amersham Biosciences, Buckinghamshire, UK). PCRs were performed by ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) with the QuantiTect SYBR Green PCR kit (Qiagen Sciences, Valencia, CA) according to the manufacturer's protocol. Primers were designed with Primer Express version 2.0 (Applied Biosystems) and were synthesized by Integrated DNA Technologies (Coralville, IA). The sequences of the primers used to detect specific genes were as follows: Plunc, 5′-TGGTCTTGTGCAGAGTCCTG-3′ (forward, nt 484), 5′-CCTCCCCTGATTGTCTTTCA-3′ (reverse, nt 643); thioredoxin reductase 2, 5′-GCTTCTGGCAAGGAAGACAC-3′ (forward, nt 938), 5′-CCCTCAGCAACATCTCCAAT-3′ (reverse, nt 1128). The relative level of mRNA expression of a specific gene was calculated based on ΔΔCT method according to the manufacturer's instruction and normalized to mRNA level for the housekeeping gene β-actin.

Results

Biochemical studies

Male C57BL/6 mice on a 3% NaCl diet were injected ip with either 10 μg/kg of aldosterone (experimental group) or vehicle (control group) and killed at the following time points after the injection: 0 h (no injection), 0.5, 1, 2, 3, 4, 5, and 12 h (five mice in each group at each time point). Plasma aldosterone and corticosterone concentrations were determined in each animal (Fig. 1). Plasma corticosterone levels increased in both groups, peaking (450 ng/ml) at 1–2 h, and returned to baseline at 4 h. In contrast, plasma aldosterone concentrations increased substantially only in the aldosterone injection group in which they reached the highest level (290 ng/dl) at 30 min, comparable with the levels seen in animals on a low-salt diet (data not shown), returning to baseline at 3 h.

Microarray analysis

Total RNA was extracted from whole hearts of every animal. RNA samples for all five animals at each time point were pooled for the purpose of the analysis.

It is known that expressed genes are more likely to have higher signal intensity (26). It has been shown that in the majority of human tissues fewer than 25% of all known genes are expressed in each tissue (27). We therefore limited data

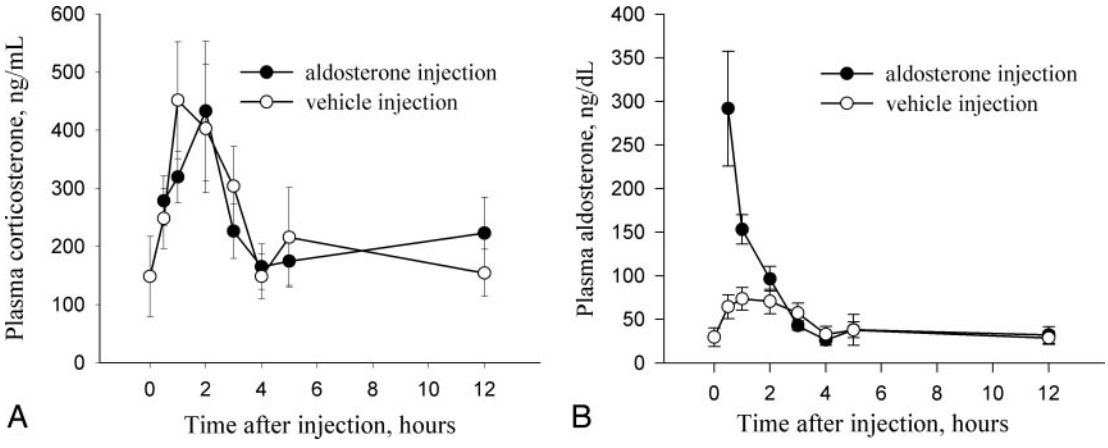

FIG. 1. Average plasma corticosterone (A) and aldosterone (B) levels over the course of the experiment. *Closed circles* represent experimental group and *open circles* represent control group of animals. *Bars* indicate SE.

TABLE 1. Coefficient of variation of signal intensity of probe sets against different gene regions

Probe set location	Actin	GADPH	Transferrin receptor	Pyruvate dehydrogenase
5′ region	0.3	0.68	0.48	0.33
Middle	0.27	0.33	0.89	0.66
3′ region	0.16	0.12	0.53	0.19

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

analysis to the probes with mean signal intensities of all 15 data points in the top 25% by magnitude (above 1280).

mRNA is usually degraded in the cell starting from the 5′ end. This is known to lead to more reliable detection of gene expression by probes against 3′ regions of the corresponding genes (28). We examined coefficient of variation of probes against 5′, middle, and 3′ regions of four constitutively expressed mouse genes routinely used as controls in Affymetrix microarrays (Table 1). Most of the time, coefficient of variation for probe sets against 3′ regions was substantially lower than coefficient of variation for either 5′ or middle region of the corresponding gene [one exception was the gene for transferrin receptor whose mRNA undergoes a unique degradation pathway starting at the 3′ end (29)]. We subsequently verified that this applies to the genes whose mean signal intensities were greater than 1280 with more than one probe set. For these genes the probe sets against 5′ regions of the genes had a higher coefficient of variation than probe sets against 3′ regions ($P < 0.0001$; Student's t test). Therefore, only data on 5111 probe sets that were either unique for the gene or the closest to the 3′ end of the mRNA of all probe sets specific for the gene on the microarray chip were used in further analysis.

To leverage the time-dependent information in this experiment, we used CAGED 1.0, a new computational tool specifically designed for hierarchical cluster analysis of temporal microarray experiments using a Bayesian approach (23). The probe sets were clustered into three distinct expression patterns (Fig. 2). The first cluster consisted of 5099 probe sets for which there was no significant difference between aldosterone injection and control groups over the course of the experiment. The second cluster consisted of three probe sets whose expression level decreased in the aldosterone injection group relatively to the control group

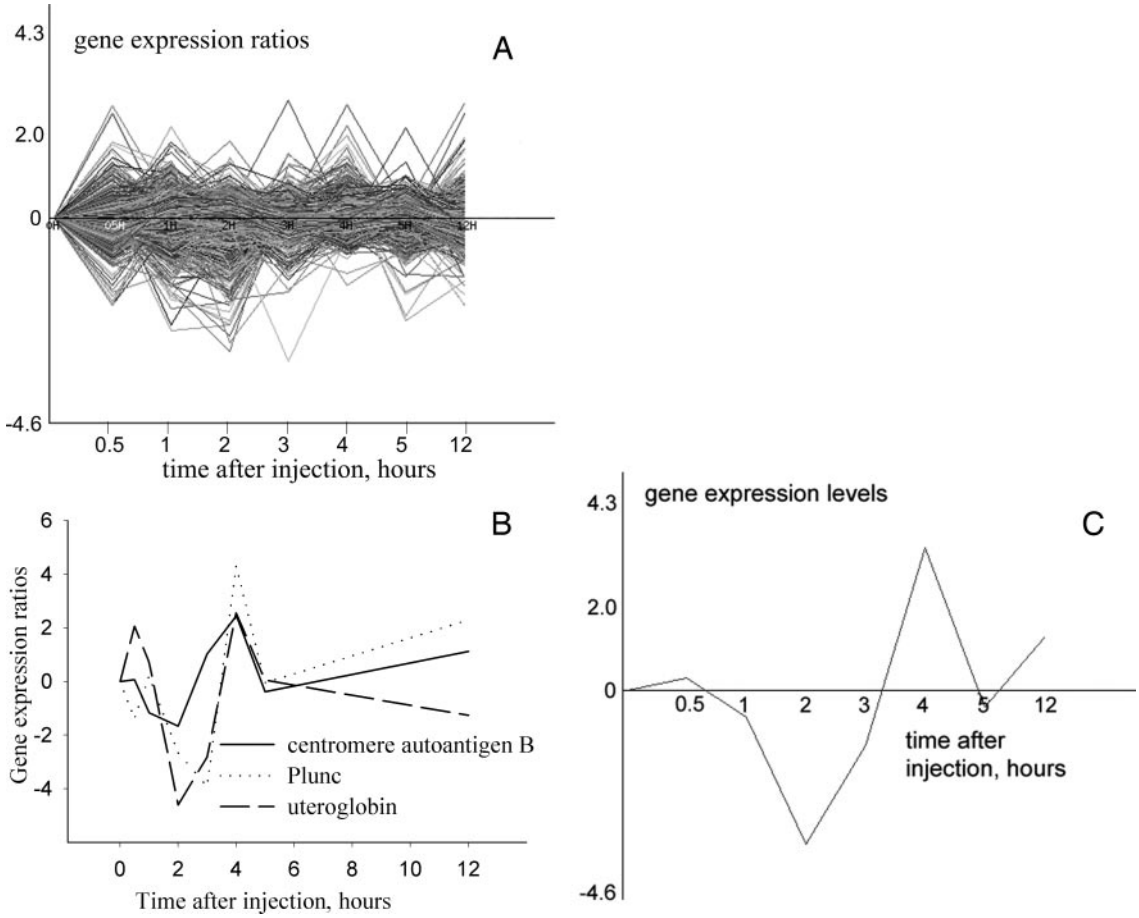

FIG. 2. Natural logarithms of the ratios of signal intensities of the genes in the aldosterone-injected mice to the signal intensities in the vehicle-injected mice (y-axis) are plotted along the timeline of the experiment (x-axis). Mean expression profile of genes with no change between aldosterone and control was 0 for all time points (data not shown). A, Individual expression profiles of the 5099 genes showing no significant change in expression level between aldosterone- and vehicle-injected groups over the course of the experiment. B, Individual expression profiles of three representative genes with a significant change in expression level between aldosterone injected and control groups over the course of the experiment. C, CAGED-generated mean expression profile of three representative genes with a significant change in expression level between aldosterone-injected and control groups over the course of the experiment.

TABLE 2. Genes determined to be differentially expressed between aldosterone injection and control groups by CAGED analysis

Gene	CAGED cluster	Function
Centromere autoantigen B	2	Cell division
Plunc	2	Unknown
Uteroglobin	2	Inhibits phospholipase A2; antiinflammatory
Nucleolar protein family 6	3	Ribosome biogenesis
Dual-specificity phosphatase 7	3	Serine/treonine phosphatase; dephophorylates ERK
Protein tyrosine phosphatase-σ	3	Development
Protein phosphatase 5	3	Negatively influences glucocorticoid receptor signaling
F-box and WD-40 domain protein 8	3	Ubiquitin protein ligase
Scavenger receptor class B, member 1	3	Mediates high-density lipoprotein uptake in liver and steroidogenic cells; regulation of steroid biosynthesis
Thioredoxin reductase 2	3	Cellular defense against reactive oxygen species
Ankyrin repeat and SOCS box-containing protein 15	3	Structural protein
Eph receptor A4	3	Receptor tyrosine kinase; development

SOCS, Suppressor of cytokine signaling.

between 1 and 3 h after the injection, then rebounded at 4 h, and came back to baseline approximately 5 h after the injection. The third cluster included nine probe sets that displayed an expression pattern similar to the ones in the second cluster but with smaller magnitude.

Genes in the second and third clusters, whose expression profiles were different between the aldosterone injection and control groups, are listed in Table 2. Phosphatases, including dual-specificity phosphatase 7, protein tyrosine phosphatase σ, and protein phosphatase 5, featured prominently in this group; other genes encoded proteins responsible for steroid biosynthesis, inactivation of reactive oxygen species, and structural proteins as well as proteins of unknown function.

Real-time RT-PCR

To verify the validity of our microarray experimentation and analysis, we performed quantitative real time RT-PCR on representative genes from the second and third CAGED clusters. As illustrated in Fig. 3, the temporal expression profiles obtained by RT-PCR closely aligned with the microarray data, confirming our results.

Discussion

In this paper we present the results of the investigation of the temporal pattern of changes in gene expression in the heart after an acute systemic administration of a physiologic dose of aldosterone.

Elucidation of early response genes for aldosterone has traditionally been difficult because the mineralocorticoid receptor binds the physiological glucocorticoids cortisol and corticosterone with affinity comparable with that of aldosterone (30, 31). There is also evidence that at least in some tissues activation of glucocorticoid receptor may induce effects similar to agonist activation of mineralocorticoid receptor (32, 33). Consequently this experiment was designed to elucidate the effects specific to aldosterone. Whereas both the aldosterone injection and control animals experienced a similar rise in plasma corticosterone levels (presumably due to the stress of the injection), the ratios of expression levels of aldosterone injection to control group for every gene at every time point were used in the data analysis, thereby canceling out the effect of corticosterone on gene expression. This experimental design also controlled for other potential confounders related to the injection which could have influenced gene expression levels.

Animal models of mineralocorticoid excess demonstrate cardiac damage (1, 7, 8), and human clinical trials offer evidence that blockade of mineralocorticoid receptor can decrease morbidity and mortality in patient with heart failure (15, 16). This experiment was designed to focus on the genetic

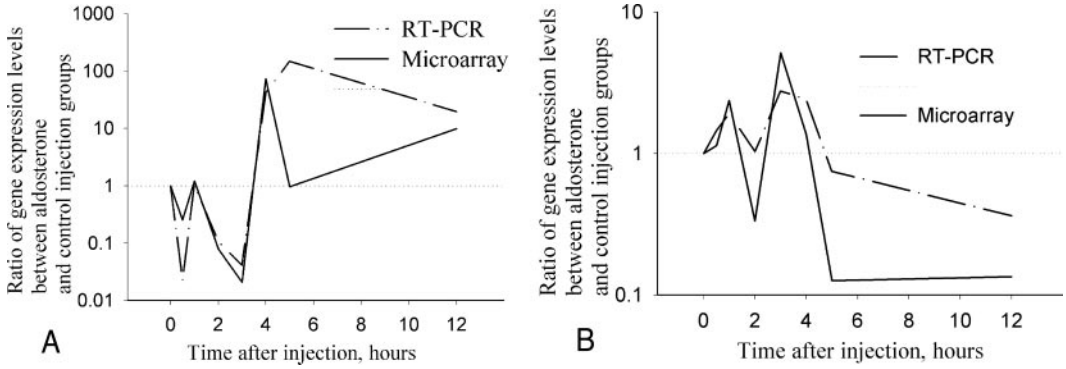

FIG. 3. Comparison of quantitative expression profiles of representative genes (Plunc in A, thioredoxin reductase 2 in B) with a significant difference in expression level between aldosterone-injected and control groups obtained by microarray analysis and real-time RT-PCR. Ratios of signal intensities of the genes in the aldosterone-injected mice to the signal intensities in the vehicle-injected mice (y-axis) are plotted along the time line of the experiment (x-axis).

effects of aldosterone that could potentially mediate empirically observed myocardial injury. In rodent models, uninephrectomy and high-salt diet are necessary for demonstration of mineralocorticoid-mediated cardiac damage (7, 12, 34). Whereas the duration of this experiment was too short to observe myocardial injury, experimental conditions (uninephrectomy, high salt diet, and high physiologic levels of aldosterone) were designed to replicate the physiological milieu that over longer periods of time has been shown to lead to cardiac damage.

In this experiment we used a novel software program CAGED to identify genes differentially expressed between aldosterone injection and control groups. CAGED was specifically designed to analyze time series microarray experiments. It employs Bayesian clustering to provide a statistically robust view of the natural dynamic structure of the transcriptome. We also used several noise-reduction strategies, including limiting analysis to microarray probes with high signal intensities (35) and probe sets closest to the 3′ end of the transcript (28). Most of the constitutively expressed mouse genes used by Affimetrix as controls had the lowest coefficient of variation in the probes located closest to the 3′ end of their mRNAs, supporting this strategy. One exception was transferrin receptor whose mRNA undergoes a unique degradation process starting at the 3′ end (29), likely leading to the relative increase in the coefficient of variation of 3′ probes observed in this experiment. The noise-reduction approach in conjunction with the use of Bayesian analysis by CAGED permitted us to identify a small number of genes with a unique pattern of expression after an acute aldosterone administration; temporal expression profiles of representative genes were subsequently verified by quantitative real time RT-PCR.

The genes found to be differentially expressed between the aldosterone injection and control groups fell into several functional categories: phosphatases; steroid biosynthesis; inactivation of reactive oxygen species (thioredoxin reductase 2); structural proteins (ankyrin repeat and suppressor of cytokine signaling box-containing protein 15); and others, including genes whose function in the heart is currently unknown (Plunc). All of the genes exhibited a similar expression pattern of suppression (relative to the control group) at 1–3 h after aldosterone administration followed by a brief rebound increase and a subsequent return to baseline.

Several of the phosphatases whose expression level was affected by aldosterone are known to play a role in steroid signaling: dual specificity phosphatase 7 dephosphorylates ERK (36), and protein phosphatase 5 may be involved in glucocorticoid receptor signaling (37). Several other groups have reported an increase in phosphorylation of ERK1/2 within several minutes after aldosterone administration that is not blocked by inhibitors of transcription and translation (38, 39), thus implying a nongenomic mechanism. Our findings possibly indicate a second, genomic pathway leading to the ERK1/2 phosphorylation (by means of decreasing the expression of the enzyme that dephosphorylates ERK1/2) after a longer lead time, potentially leading to a more sustained effect.

Another important group of genes affected by aldosterone were regulators of tissue damage and inflammation: thioredoxin reductase 2 provides cellular defense against reactive oxygen species (40), and uteroglobin (also known as blastokinin, secretoglobin family 1A, member1, or Clara cell secretory protein) inhibits phospholipase A2 and therefore potentially has an antiinflammatory effect (41). Excess of aldosterone was known to cause inflammatory lesions in the heart as long as more than half a century ago (1). More recently ours and other laboratories have shown that aldosterone and other mineralocorticoids induce inflammatory markers and subsequently lead to development of histologic picture of inflammation in the coronary vessels (12, 34, 42). These effects can be duplicated by administration of 11β-hydroxysteroid dehydrogenase type 2 inhibitors (42). Our findings of a decrease in the expression level of thioredoxin reductase and uteroglobin after aldosterone administration suggest a possible mechanism for these responses.

Multiple investigators have reported cardiac fibrosis as one of the prominent effects of concomitant exogenous aldosterone administration and a high-salt diet (9, 13, 43). Interestingly, however, no cardiac fibrosis was found in transgenic mice expressing increased levels of aldosterone synthase in the heart leading to elevated intracardiac aldosterone concentration (44). This suggests that development of cardiac fibrosis may be a secondary rather than primary effect of aldosterone. In our experiment we did not find any evidence of increased expression of enzymes involved in collagen synthesis, consistent with this hypothesis.

The experimental design of the study had its limitations. Because corticosterone levels increased in both control and experimental animals, we would not be able to detect a change in the expression levels of genes influenced by both glucocorticoid and mineralocorticoid receptors, most notably serum and glucocorticoid-inducible kinase (45). This may account for the absence of some genes whose expression level was increased by aldosterone. However, serum and glucocorticoid-inducible kinase expression levels in the heart have not been shown to be affected by aldosterone (46). Consequently, we felt that it was important to first identify genetic mechanisms unique to aldosterone, which then could serve as a foundation for further investigation into the complexity of genetic response to mineralocorticoids.

RNA samples in this experiment were pooled (five animals per microarray) to increase the statistical power. The power could be increased further if each individual animal's RNA sample was analyzed on its own microarray chip and potential outlier values identified. However, statistical validity of using pooled RNA samples for microarray analysis is well established (47). Importantly, the data at all time points in the experiment were congruent, making it highly unlikely that a single outlier value could have significantly influenced the findings.

In this exploratory study, we deliberately chose an *in vivo* model of mineralocorticoid excess that closely replicated experimental conditions that were previously reported to lead to cardiac injury. The study was not designed to identify the effect of aldosterone on specific tissues and cells in the heart. This should be the subject of further *in vitro* studies that will build on the *in vivo* findings reported here. It will also be important to establish the role of mineralocorticoid receptor in mediating these effects of aldosterone. Investigation of the

effects of long-term, rather than short-term, aldosterone exposure on the gene expression in the heart may shed more light on the mechanisms of observed aldosterone-mediated cardiac damage.

In summary, the current study presents the results of an investigation of the timeline of changes in gene expression profile in the heart after acute *in vivo* aldosterone administration. We used a novel software program, CAGED, to identify a small number of genes whose expression patterns were altered in the aldosterone-injected animals. The known functions of a number of these genes, which have not been previously reported to be affected by aldosterone, are consistent with known effects of aldosterone at both molecular and histologic level.

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