Gene Expression Profiles of Peripheral Blood Mononuclear Cells Reveal Transcriptional Signatures as Novel Biomarkers of Cardiac Remodeling in Rats With Aldosteronism and Hypertensive Heart Disease

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Objectives
In searching for a noninvasive surrogate tissue mimicking the pro-oxidant/proinflammatory hypertensive heart disease (HHD) phenotype, we turned to peripheral blood mononuclear cells (PBMCs). We tested whether iterations in \([Ca^{2+}]_i\), \([Zn^{2+}]_i\), and oxidative stress in cardiomyocytes and PBMCs would complement each other, eliciting similar shifts in gene expression profiles in these tissues demonstrable during the preclinical (week 1) and pathological (week 4) stages of aldosterone/salt treatment (ALDOST).

Background
Inappropriate neurohormonal activation contributes to pathological remodeling of myocardium in HHD associated with aldosteronism. In rats receiving long-term ALDOST, evidence of reparative fibrosis replacing necrotic cardiomyocytes and coronary vasculopathy appears at week 4 associated with the induction of oxidative stress by mitochondria that overwhelms endogenous, largely Zn\(^{2+}\)-based, antioxidant defenses. Biomarker-guided prediction of risk before the appearance of cardiac pathology would prove invaluable.

Methods
In PBMCs and cardiomyocytes, quantitation of cytoplasmic free Ca\(^{2+}\) and Zn\(^{2+}\), \(H_2O_2\), and 8-isoprostane levels and isolation of ribonucleic acid (RNA) and gene expression together with statistical and clustering analyses and confirmation of genes by in situ hybridization and reverse-transcription polymerase chain reaction were performed.

Results
Compared with controls, at weeks 1 and 4 of ALDOST, we found comparable increments in \([Ca^{2+}]_i\), \([Zn^{2+}]_i\), and 8-isoprostane coupled with increased \(H_2O_2\) production in cardiac mitochondria and PBMCs, together with the common networks of expression profiles dominated by genes involved in oxidative stress, inflammation, and repair. These included 3 central Ingenuity pathway-linked genes: p38 mitogen-activated protein kinase, a stress-responsive protein; nuclear factor-\(\kappa B\), a redox-sensitive transcription factor and a proinflammatory cascade that it regulates; and transforming growth factor-\(\beta_1\), a fibrogenic cytokine involved in tissue repair.

Conclusions
Significant overlapping demonstrated in the molecular mimicry of PBMCs and cardiomyocytes during preclinical and pathological stages of ALDOST implies that transcriptional signatures of PBMCs may serve as early noninvasive and novel sentinels predictive of impending pathological remodeling in HHD. (J Am Coll Cardiol HF 2013;1:469–76) © 2013 by the American College of Cardiology Foundation

The pathophysiological origins of the heart failure associated with hypertensive heart disease (HHD) are rooted in neurohormonal activation that includes circulating effector hormones of the renin-angiotensin-aldosterone system (1,2).

See page 477

It is the endocrine actions of the renin-angiotensin-aldosterone system circulating hormones that account for
a systemic response the major features of which include a pro-oxidant phenotype involving diverse tissues, in which the rate of reactive oxygen species generation overwhelms endogenous antioxidant defenses consisting of crucial Zn$^{2+}$-dependent enzymes (3,4); an immunostimulatory state, in which activated circulating lymphocytes and monocytes (peripheral blood mononuclear cells [PBMCs]) elaborate proinflammatory cytokines (5–9), and a catabolic state with proteolytic degradation of skeletal muscle and elevations in circulating parathyroid hormone (PTH) that account for bone resorption and predispose to fracture (10–15). The failing heart likewise undergoes a progressive hormone-mediated structural and biochemical remodeling with ongoing loss of cardiomyocytes.

Biomarkers that, during a preclinical stage, would reveal risk before as well as the presence of cardiac pathology would advance the overall evaluation and management of patients having heart failure with hypertension (HHD) and to monitoring cardiac remodeling. In searching for a surrogate tissue, tracking the pro-oxidant/proinflammatory pathway to cardiomyocyte necrosis, we turned to PBMCs (lymphocytes and monocytes) as integral to cellular/molecular mechanisms of tissue repair and cardiac pathology (18). Our rationale in targeting PBMCs as a putative surrogate was based on the following: 1) the gene expression profile of multiple tissues is shared with PBMCs (19); 2) PBMCs are integral to the neuroendocrine–immune system interface invoked during stressor states with the microarray profiling of their transcriptome used to identify molecular signaling pathways; 3) PBMC activation reveals molecular signatures in chronic heart failure and predicts its severity (5–9,20,21); 4) our previous studies identified the activation of the PBMC transcriptome at preclinical (week 1) and pathological (week 4) stages of aldosterone/salt treatment (ALDOST) in rats (22,23). Cardioprotection, in turn, was assessed by monitoring temporal iterations in cation composition, redox status, and an altered PBMC transcriptome (22–25). Hence, these cells lend themselves as potential biomarkers of noninvasive and serial sampling during preclinical and pathological stages of heart failure with hypertension (HHD) and to monitoring cardioprotective strategies (3–9,25). We hypothesized that the pathophysiological changes seen with ALDOST are potentiated by integrated interactions between hormones and divalent cations (i.e., Ca$^{2+}$ and Zn$^{2+}$ as pro-oxidant and antioxidant), and that these iterations will cause unique and yet similar shifts in gene expression in heart tissue and PBMCs, thus providing characteristic molecular and transcriptional signatures.

**Methods**

**Animal model.** Eight-week-old male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, Indiana) were used as previously reported (22,23). They received aldosterone (0.75 µg/h) via an implanted minipump (Alzet, Cupertino, California) together with 1% NaCl/0.4% KCl in drinking water (ALDOST), and standard laboratory chow (Teklad 2215 Rodent Diet, Harlan Laboratories, Madison, Wisconsin) for 1 and 4 weeks (6 each group). Six unoperated on, untreated age-/sex-matched rats served as controls. This study was approved by the institution’s Animal Care and Use Committee. The investigation conforms to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health.

**PBMCs and cardiomyocyte Ca$^{2+}$, Zn$^{2+}$, H$_2$O$_2$, and 8-isoprostan.** Quantitation of divalent cations H$_2$O$_2$ and 8-isoprostane was performed, as previously described (23,25).

**Isolation of ribonucleic acid (RNA) and gene expression analysis.** We isolated RNA using Trizol Reagent (Life Technologies, Carlsbad, California), as previously described (26). The RNA underwent gene expression analysis using Affymetrix software (Affymetrix Inc., Santa Clara, California), as previously described (22). This analysis was conducted by the Molecular Resource Center Core Lab at our institution using the GCOS 1.4 software from Affymetrix Inc. We previously reported on the efficacy of our core facility to replicate the relevant expression array data by quantitative reverse-transcription polymerase chain reaction (RT-PCR).

**Statistical and clustering analysis of gene expression data.** Expression data files were uploaded into the GeneSpring DX software (Agilent Technologies, Santa Clara, California) for statistical and cluster analysis, as previously described (27). Light filtering was initially used to remove those probe sets that were not present in at least 1 of the samples, followed by a 1-way analysis of variance (ANOVA) ($p < 0.05$) with Benjamini–Hochberg correction for multiple hypothesis testing. Hierarchical clustering of the genes from our ANOVA was carried out to further sort those into subgroups with specific expression patterns, depending on their up- or down-regulation in response to ALDOST.

**Data mining of gene probe sets identified by the data analysis.** The main data mining tool used was Ingenuity Pathways Analysis (Ingenuity Systems, Inc., Redwood City,
California), a subscription-based, Web-delivered (www.ingenuity.com) application that allows users to discover, visualize, and explore networks relevant to their experimental results. Our use of this pathway analysis approach was previously described in detail (27). The pathway analysis not only serves as a tool to gain insights but also to eliminate false-positive genes because random genes (such as false positives) are much less likely to connect to pathways than genes that are changed due to shifts in biological processes and biochemical pathways.

**Confirmation of genes by in situ hybridization and RT-PCR.** We used RT-PCR and in situ hybridization as alternative approaches to evaluate and confirm gene expression in heart tissue. RT-PCR was conducted on total RNA extracted from cardiac tissue using Trizol Reagent (Invitrogen, Carlsbad, California) by methods described previously (28). Gene-specific probes and primer sets were deduced using Universal ProbeLibrary Assay Design software (Roche Applied Science, Indianapolis, Indiana). In situ hybridization of sections (16 μm) fixed in 4% formaldehyde was conducted, as previously described (29).

**Results**

**Intracellular Ca\(^{2+}\) and Zn\(^{2+}\).** Cytosolic free [Ca\(^{2+}\)]\(_i\) and [Zn\(^{2+}\)]\(_i\) concentrations were determined in PBMCs and cardiomycocytes. An approximate doubling of these divalent cations was found at 1 week and did not change appreciably at 4 weeks of ALDOST. The magnitude of these changes was similar in both PBMCs and cardiomycocytes, albeit the absolute amount of cytosolic free Ca\(^{2+}\) and Zn\(^{2+}\) differs between these respective noncontractile and contractile cells (Table 1).

**Oxidative stress and free radical–induced damage.** An increase in intracellular [Ca\(^{2+}\)]\(_i\) and intramitochondrial [Ca\(^{2+}\)]\(_m\) stimulates the generation of H\(_2\)O\(_2\) by these organelles and consequent free radical–induced damage to lipids determined by increased levels of 8-isoprostane. Both PBMCs and mitochondria demonstrated a significant increase in H\(_2\)O\(_2\) generation at 1 week and with a further increase at 4 weeks of ALDOST and an increase in 8-isoprostane levels in both PBMCs and cardiomycocytes at 1 week and 4 weeks (Table 2).

**Gene expression profiles of the heart.** We examined gene expression in heart tissue collected after 1 and 4 weeks of ALDOST and compared them with controls, using the Affymetrix Rat 230 2.0 expression array. Of the 31,099 probe sets on this array, 22,538 showed expression (present or marginal signal) in at least 1 of the 15 samples. We conducted ANOVA on the 22,538 probe sets to define genes that were affected by ALDOST (Benjamini-Hochberg multiple test correction \(p \leq 0.01\)) and identified 83 probe sets (Online Table 1). Hierarchical clustering indicated that each of these probe sets belonged to 1 of 2 expression patterns that either quenched or amplified in response to ALDOST (Fig. 1A). However, it must be emphasized that the effect of ALDOST was already evident at preclinical 1 week.

The list of probe sets clearly affected by ALDOST was systematically mined for pathway connectivity using the Ingenuity database. The 83 probe sets represented 52 different genes for which the Ingenuity knowledge database had information to connect these genes to other genes in the database. Six of these genes, however, could not be connected to any other genes from the list of 52. The remaining 46 genes were connected in 4 networks with 13, 12, and 8 of these genes belonging to each cluster. The 3 networks with \(>10\) ALDOST–associated genes in each were fused into a single network using the merge network function with the resulting network shown in Figure 1B. These 3 major networks were linked to pro-oxidant/proinflammatory-related genes NFKB, p38 MAPK, and TGF-\(\beta\).

**Gene expression profiles in PBMCs.** We analyzed gene expression in PBMCs collected from ALDOST in a manner similar to that described for heart tissue. We used the Affymetrix RAE 230A expression array and found that 10,352 of the 15,923 probe sets on this array showed expression (present or marginal signal) in at least 1 of the 15 samples. We conducted ANOVA on the 10,352 probe sets to define genes that were affected by ALDOST using

### Table 1 Cytosolic Free [Ca\(^{2+}\)]\(_i\) and [Zn\(^{2+}\)]\(_i\) in PBMCs and Cardiomycocytes

<table>
<thead>
<tr>
<th>Groups</th>
<th>PBMCs</th>
<th>Cardiomycocytes</th>
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<tbody>
<tr>
<td>[Ca(^{2+})](_i), nM</td>
<td>41.8 ± 3.7</td>
<td>29 ± 4</td>
</tr>
<tr>
<td></td>
<td>70.7 ± 6.2*</td>
<td>70 ± 5*</td>
</tr>
<tr>
<td></td>
<td>90.8 ± 8.1</td>
<td>80 ± 5*</td>
</tr>
<tr>
<td>[Zn(^{2+})](_i), nM</td>
<td>0.080 ± 0.001</td>
<td>0.76 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>0.175 ± 0.022</td>
<td>1.77 ± 0.09*</td>
</tr>
<tr>
<td></td>
<td>0.171 ± 0.026</td>
<td>1.64 ± 0.08*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; \(* p < 0.05\); \(\leq 0.001\) ALDOST vs. controls; 6 in each group. \(\leq p < 0.01\).

ALDOST = aldosterone/salt treatment; PBMCs = peripheral blood mononuclear cells.

### Table 2 H\(_2\)O\(_2\) Production in PBMCs and Cardiac Mitochondria and 8-Isoprostane Levels in PBMCs and Cardiomycocytes

<table>
<thead>
<tr>
<th>H(_2)O(_2)</th>
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<th>Mitochondria, pmoles/mg protein/min</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Groups</td>
<td>(Mean Channel Brightness)</td>
</tr>
<tr>
<td>PBMCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>61.0 ± 6.3</td>
<td>91.0 ± 13.4</td>
</tr>
<tr>
<td>ALDOST wk 1</td>
<td>95.0 ± 9.1*</td>
<td>118.3 ± 8.4</td>
</tr>
<tr>
<td>ALDOST wk 4</td>
<td>173.8 ± 18.5*</td>
<td>157.6 ± 13.2*</td>
</tr>
<tr>
<td>8-Isoprostane</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBMCs, pg/mg protein</td>
<td>Cardiomycocytes, pg/mg protein</td>
</tr>
<tr>
<td>Control</td>
<td>162.3 ± 25.4</td>
<td>32.8 ± 5.8</td>
</tr>
<tr>
<td>ALDOST wk 1</td>
<td>259.9 ± 37.8*</td>
<td>46.4 ± 9.5</td>
</tr>
<tr>
<td>ALDOST wk 4</td>
<td>214.6 ± 22.9*</td>
<td>341.8 ± 50.1*</td>
</tr>
</tbody>
</table>

Values are mean ± SD; \(* p < 0.001\) ALDOST vs. controls; 6 in each group. \(\leq p < 0.05\).

Abbreviations as in Table 1.
a more stringent p value of ≤0.001, but did not apply a multiple test correction to this dataset. This analysis revealed that 55 probe sets were affected by the treatment (Online Table 2). We conducted a hierarchical clustering analysis of these 55 probe sets with the resulting gene tree shown in Figure 2A. The genes that belong to subclusters...
Figure 2  Heat Map of Gene Expression Proﬁles in PBMC From Control and Aldosterone-Salt Treated Rats

(A) Heat map of the gene expression for the 3 groups. Results from hierarchical clustering of the 55 probe sets in peripheral blood mononuclear cells (PBMCs) affected by aldosterone/salt treatment (ALDOST). The solid bars on the right indicate the 42 probe sets that follow a clear pattern of increase or decrease in expression after 1 week of ALDOST. Red indicates relatively high expression, whereas blue indicates relatively low expression. (B) Network created by the Ingenuity server from the 42 genes in PBMCs shown in A. The gray nodes indicate genes that are signiﬁcantly affected by ALDOST, whereas the white icons represent genes, gene families, and molecules that are needed to connect those ALDOST-affected genes to each other in a network.
with a clear pattern of decrease or increase in response to ALDOST are noted with a solid bar to the right of the gene tree. It should be noted that for all of these 42 genes whose expression was affected by ALDOST, the responses appeared greater at 1 week, long before cardiac pathology was found.

The list of 42 probe sets, consisting of 27 different genes, clearly affected by ALDOST was mined for pathway connectivity using the Ingenuity database. Four of these genes could not be connected to any other genes from the list of 27. The remaining 23 genes were connected in 2 networks (with 12 and 11 genes in each) that were fused into a single network shown in Figure 2B. As in heart tissue, the network in PBMCs was linked to pro-oxidant/proinflammatory-related genes NFκB, p38 MAPK, and TGF-β1.

Gene expression by in situ hybridization and RT-PCR. To confirm the central role of TGF-β1 in the genesis of remodeling observed at sites of fibrosis, we conducted in situ hybridization on coronal sections and RT–PCR analysis on RNA extracted from heart tissue. The results are shown in Figure 3. In situ hybridization demonstrated a more intense signal for this cytokine in hearts of rats receiving ALDOST (Fig. 3B) compared with tissue harvested from controls (Fig. 3A). Further confirmation of this response was demonstrated by RT–PCR with a significant up-regulation of TGF-β1 in rats receiving ALDOST (Fig. 3C).

Discussion

We hypothesized that marked cation dyshomeostasis and oxidative stress induced by ALDOST will affect both the myocardium and PBMCs and reveal similar biochemical changes and gene expression profiles at preclinical and pathological stages of cardiac remodeling in HHD. Our findings imply that the PBMC transcriptome can serve as a surrogate marker of pathological events in the heart.

Increased concentrations of cytosolic free \([Ca^{2+}]_i\) and \([Zn^{2+}]_i\) were demonstrated in both PBMCs and cardiomyocytes during weeks 1 and 4 of ALDOST. Activation and induction of oxidative stress by these immune cells are known to be Ca\(^{2+}\) dependent, whereas cytokine up-regulation is Zn\(^{2+}\) dependent. Increased intracellular Ca\(^{2+}\) and Zn\(^{2+}\) levels are driven by PTH and up-regulation of metallothionein, a Zn\(^{2+}\)-binding protein (30). Ca\(^{2+}\) and Zn\(^{2+}\) are integral to regulating oxidative stress and antioxidant defenses in cardiomyocytes and PBMCs, respectively. PTH-mediated excessive \([Ca^{2+}]_i\) accumulation in PBMCs with induction of oxidative stress during mineralocorticoid/salt treatment can be prevented by parathyroidectomy (31), Ca\(^{2+}/Mg^{2+}/\text{vitamin D}_3\) supplementation (32), cotreatment with a calcium channel blocker (24), or cotreatment with spironolactone, which prevents secondary hyperparathyroidism (33). Increments in \([Zn^{2+}]_i\)-based antioxidant defenses and corresponding cardioprotection are conferred by ZnSO\(_4\) supplementation (34–36), a Zn\(^{2+}\) ionophore (35), or a β\(_3\) receptor agonist, which leads to endothelial nitric oxide (NOS) synthase–based generation of NO and NO-induced release of inactive Zn\(^{2+}\) bound to metallothionein (37,38). We found unequivocal evidence of
increased H$_2$O$_2$ production and consequent lipid peroxidation in both cardiomyocytes and their mitochondria, as well in PBMCs during the preclinical stage.

To gain more specific and target tissue–based biomarkers, Affymetrix expression arrays were used to evaluate the comprehensive gene expression patterns in heart tissue and PBMCs. Hierarchical clustering of genes eliciting a statistically significant differential expression revealed that almost all of the effects of ALDOST were already present at 1 week, and at 4 weeks, their expression remained, by and large, similar to that at 1 week.

To better illustrate the underlying processes of gene expression abnormalities induced by ALDOST, we subjected the lists of differentially expressed genes to network analysis. The network created by genes whose expression in heart tissue is significantly affected by ALDOST was dominated by genes involved in oxidative stress, inflammation, and cell growth, as well as a large number of connections to other genes related to growth factors, such as insulin, vascular endothelial growth factor, tumor necrosis factor $\alpha$, and platelet-derived growth factor.

Using cardiac tissue and PBMC transcriptomes, we unraveled a similar signal-transducer–effector pathway to be involved that includes the 3 major signaling cascades: p38 mitogen–activated protein kinase, a stress responsive protein; nuclear factor $\kappa$B (NF$\kappa$B), a downstream redox-sensitive transcription factor and its proinflammatory target genes (e.g., ICAM-1, MCP-1, and TNF-$\alpha$); and tissue repair via transforming growth factor $\beta_1$, a fibrogenic cytokine. However, we would not speculate on complete concordance of gene expression profiles between noncontractile immune cells and contractile cardiomyocytes during stressor states. We recently reported on similarities and differences in gene profiling between 2 contractile tissues, heart and muscle, in response to 4 weeks of ALDOST (39).

As demonstrated by in situ hybridization to detect and localize messenger ribonucleic acid expression and immunohistochemical labeling of protein in our previous studies (18), the network created by the PBMC transcriptome was significantly affected by ALDOST and dominated by genes involved in oxidative stress, inflammation, and cell growth and death. Central to these networks, together with a large number of connections to other genes, we found genes integral to the process of inflammation, such as IL-$\alpha$, NF$\kappa$B, and $p38$ MAPK, and growth factors, such as hepatocyte growth factor and transforming growth factor $\beta_1$ to be involved in the preclinical stage. Furthermore, the network contains a number of caspases and apoptosis-inhibiting genes, such as $STAT3$. Using the PBMC transcriptome as biomarkers in patients with decompensated heart failure, transcripts related to inflammation and oxidative stress were expressed in abundance, including NF$\kappa$B, MCP-1, tumor necrosis factor $\alpha$, and transforming growth factor $\beta_1$ (3–9). It was recently suggested that PBMC gene expression profiling may identify the preclinical stage of systolic heart failure (21), and the up-regulated PBMC gene network may provide a molecular signature distinguishing patients with heart failure from healthy controls (8).

Our investigation of gene expression profiles in heart tissue and PBMCs indicated that although there are similarities in the molecular pathways activated by ALDOST, differences also exist. These differential expression patterns are perhaps due to the fact that the 2 processes are played out somewhat differently in these 2 tissues. PBMCs are the effectors of inflammation where the coronary vasculature is one of their targets. Within the networks created from the 2 different tissues, we identified 3 central genes that are common: $p38$ MAPK, NF$\kappa$B, and TGF-$\beta_1$. We previously showed that NF$\kappa$B plays a central role in the proinflammatory cardiac phenotype seen at week 4 ALDOST, together with the proinflammatory genes it regulates (18). In those studies, NF$\kappa$B and 3-nitrotyrosine, an index of oxidative damage, were shown to be amplified in the heart by immunohistochemistry. Both NF$\kappa$B and p38 mitogen-activated protein kinase are redox sensitive and activated by reactive oxygen species. Cotreatment with either N-acetylcysteine, an antioxidant, or a Zn$^{2+}$ ionophore attenuated these responses, implicating the relevance of antioxidant defenses (18).

**Study limitations.** We did not obtain serial blood samples from the same animal at weeks 1 and 4 of ALDOST, nor did we investigate all transcriptome findings in heart tissue with RT-PCR methodology. We did, however, confirm the gene chip data and central Ingenuity pathway analysis with our earlier interrogation of mRNA expression and up-regulated proteins using in situ hybridization and immunohistochemistry, respectively (18). Furthermore, the use of pathway analysis can eliminate false-positive genes because those genes are much less likely to connect in pathways than true positives affected by changes in biological processes. Finally, we did not conduct a prediction analysis, as suggested by Heidecker et al. (40).

**Conclusions**

[Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_m$ overloading of cardiomyocytes and PBMC [Ca$^{2+}$]$_i$ occur, together with the induction of oxidative stress, in rats during ALDOST. There is a similarity in the transcriptome of PBMCs and heart tissue that is first evident during the preclinical stage and persists into the pathological stage of chronic aldosteronism with HHD. These findings raise the prospect that PBMCs are capable of revealing the early diagnostic signs as a noninvasive biomarker expression profile predictive of cardiac remodeling and thereby has the potential to serve as a novel sentinel and useful molecular signature in monitoring cardioprotection and management strategies in human HHD.

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REFERENCES


Key Words: aldosteronism | calcium | cardiomyocytes | gene chip array | oxidative stress | peripheral blood mononuclear cells | zinc.

APPENDIX

For supplemental tables, please see the online version of this article.