NADPH Oxidase Inhibition in HeartFailure ImprovedVascular Function Associated withChanges in the Novel Genes Expression Revealed byTranscriptome Analysis

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Abstract
Vascular endothelium-dependent vasorelaxation is diminished and reduced skeletal muscle blood flow and correlates with the severity of symptoms in heart failure (HF) as a result of the significant elevation of superoxide anion (O2•−) production. There are several sources of (O2•−) production within vessels, but NADPH oxidase is present in vascular smooth muscle cells and endothelial cells. Therefore oxidative stress may attenuate endothelial function and inhibition of this action may become one of the strategies to treat HF. We previously investigated the global transcriptome analysis in tachycardia induced HF dogs and we selected four core genes, SOCS3, GADD45A, CDKN1A, and DUSP5 which were associated with the p53 pathway-related genes and the inflammatory interleukin-related genes enhanced expression in HF. We examined therapeutic effects of apocynin (0.3 mg/kg/day) which suppressed generation of (O2•−) on vascular endothelial function and those gene expressions in the femoral artery. Apocynin significantly increased % femoral blood flow responses by acetylcholine (HF 196.4 ± 24.7% vs. apocynin 342.2 ± 35.4%, P<0.05), suppressed O2•− production (HF 17.9 ± 1.9 LU/mg/min vs. apocynin 12.89 ± 1.6 LU/mg/min, P<0.05) and NADPH oxidase activity (HF 124.9 ± 20.4 RLU/mg/min vs. apocynin 63.9 ± 14.7 RLU/mg/min P<0.05) in HF. The agent decreased the levels of SOCS3, GADD45A, CDKN1A and DUSP5 mRNAs expressions. Suppression of oxidative stress improved endothelial dysfunction in HF through pathways closely linked with cell cycles, proliferation, apoptosis and inflammation. We can conclude that the specific inhibition of NADPH oxidase will become one of the promising therapeutic targets in the treatment of HF mediating through novel vascular molecular mechanisms.

Keywords: NADPH oxidase; Heart failure; Transcriptome analysis; Apocynin

Introduction
Vascular endothelium-dependent vasorelaxation is diminished in heart failure (HF) and oxidative stress plays a pivotal role in the alteration of endothelial function in HF [1]. NADPH oxidase is an inducible electron transport system and represents the most important source of superoxide anion (O2•−) production in vascular cells [2]. This enzyme mediated endothelial-dependent vasodilatation in HF was attenuated, accompanied by upregulation of the expression of Nox2 and p47phox, which are NADPH oxidase subunits as we previously reported [3]. However, the etiology of vascular endothelial dysfunction in HF is multifactorial and a variety of mediators are involved in its progression. The elucidation of transcriptome complexity and understanding the underlying functions of various differentially expressed genes is particularly useful in assessing the possible effects of disease states or treatment conditions on the transcriptome [4].

We identified changes in gene expression strongly related to occurrence of HF using global transcriptome analysis of next-generation sequencers and revealed that expression of the p53 pathway-related genes and the inflammatory interleukin-related genes showed enhanced expression in HF compared with the normals [5]. p53, a transcription factors, which is involved in many biological processes, including cell cycle arrest, DNA repair, senescence and apoptosis has also been linked to redox homeostasis. Reactive oxygen species (ROS) can modify the cysteine residues of p53, leading to a conformational change that affects its transcriptional activities [6]. IL-6 is an inflammatory cytokine that appears to play a key role in the activation/maintenance of the inflammatory response in chronic disease including HF and IL-6 levels are independent risk factors for mortality in patients with HF as we previously reported [7]. Because it still remains unclear as to what extent chronic NADPH oxidase inhibition improve vascular endothelial dysfunction in HF and effect on the functional significant genes whose expression was most highly up-regulated in HF. We assessed the impact of therapeutic intervention using a specific NADPH oxidase inhibitor, apocynin on the vascular dysfunction and the gene expressions responsible for NADPH oxidase in tachycardia-induced HF dogs.

Materials and Methods
Animal preparation
All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, National Institutes of Health, Publication no. 86-23) and approved by the Committee of the Research Center for Animal Life Science at Shiga University of Medical Science (Approval No. 2003-7-16). After anaesthesia was induced using pentobarbital sodium (25 mg/kg), animal preparation was performed and HF was induced.
Experimental protocol

Effects of apocynin on hemodynamics and endothelium-dependent vasodilation in HF

Animals were divided into three groups in a randomized fashion:
- The NADPH oxidase inhibition group (n=5) received apocynin (0.3 mg/kg/day; orally once daily).
- The HF group (n=5) received only vehicle.
- The normal group (n=5) underwent the same procedure without pacing.

On the 29th day after the initiation of pacing, the pacemaker was deactivated and arterial blood pressure and echocardiographic measurements were subsequently performed as described previously [9]. To evaluate endothelium-dependent vasodilation, all dogs were given thiopental sodium to provide conscious sedation and allowed to breathe spontaneously during the procedure.

A Doppler flow probe was placed on the femoral artery to measure femoral blood flow (FBF) as previously described [3]. After the administration of indomethacin to block the cyclooxygenase pathway, the endothelium-dependent vasodilator acetylcholine (ACh) at a dose of 0.1 μg/kg/min was infused into the femoral artery. FBF, aortic pressure (AoP) and heart rate (HR) were monitored continuously and recorded. Peak responses of FBF to ACh were used for analysis.

Preparation of aortic tissues

The dogs were anesthetized with a large dose of pentobarbital sodium and euthanized by bleeding. The segments of aorta (5 mm) were placed into chilled modified Krebs/HEPES buffer and some were frozen in liquid nitrogen [3].

Measurement of ex vivo vascular superoxide anion (O₂⁻) formation

The O₂⁻ production and NADPH oxidase activity in the aortic segments were measured using the lucigenin-enhanced chemiluminescence method as previously reported [3].

Quantitative reverse-transcription-polymerase chain reaction (qRT-PCR)

For the analysis of canine aortic suppressor of cytokine signaling 3 (SOCS3), growth arrest and DNA damage 45A (GADD45A), cyclin-dependent kinase inhibitor 1 A (CDKN1A) and dual specificity protein phosphatase 5 (DUSP5) gene expression, total RNA was extracted from the frozen aorta and quantitative RT-PCR was performed as described previously [5]. Gene expression was normalized using the GAPDH gene. Canine-specific primer sequences are followed; SOCS3 (Forward 5' to 3'); AGGAGAGCGGCTTCTACTGGAG, Reverse (5' to 3'); GGGTGTGCCTGGCACCTCTC), DUSP5 (Forward; TCCTCAAA GGGGATATGAG, Reverse; GGGGATATGAGAC, Reverse; GGTTGGCGAGGAACTCGCAC). All quantification analyses were performed in triplicate.

Table 1: Effects of apocynin on hemodynamics and femoral blood flow.

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=5)</th>
<th>HF (n=5)</th>
<th>Apocynin (n=5)</th>
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</thead>
<tbody>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>124.1 ± 4.9</td>
<td>94.2 ± 7.1</td>
<td>90.7 ± 6.7³</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>125.1 ± 10.6</td>
<td>164.5 ± 11.2</td>
<td>171.2 ± 14.1¹</td>
</tr>
<tr>
<td>%Fractional shortening</td>
<td>32.5 ± 1.1</td>
<td>11.9 ± 1.3</td>
<td>12.4 ± 2.1</td>
</tr>
<tr>
<td>FBF (mL/min)</td>
<td>5132.6 ± 290.7</td>
<td>1829.1 ± 287.5</td>
<td>1995.1 ± 111.1¹</td>
</tr>
<tr>
<td>% increase in FBF by ACh</td>
<td>594.7 ± 75.2</td>
<td>196.4 ± 24.7⁰</td>
<td>341.2 ± 35.4¹</td>
</tr>
</tbody>
</table>

FBF: Femoral Blood Flow; ACH: Acetylcholine. Values are mean ± SEM. *P<0.05 compared with the normal group, † P<0.05 compared with the HF group.

Statistical analysis

Data are expressed as mean ± SEM. Differences between two groups were assessed by Student’s t test when appropriate. Differences among three groups were assessed by ANOVA with Scheffe’s-F test when appropriate. A P-value of 0.05 was chosen as the cut-off significance level.

Results

Cardio-hemodynamics data

As shown in Table 1, after 4 weeks of pacing, the mean aortic pressure and fractional shortening decreased and HR increased in the HF group compared with the normal group and those values did not differ significantly between the HF group and the apocynin group. Baseline FBF was significantly decreased in both the HF and apocynin groups compared with the normal. In addition, the percent ratio of the ACh-induced FBF to baseline FBF was significantly impaired in the HF group compared with the normal group, but the % changes was significantly enhanced by apocynin when compared with the HF group.

Effects of apocynin on vascular O₂⁻ formation and NADPH oxidase activity in HF

As shown in Figure 1, O₂⁻ formation and NADPH oxidase activity in the aorta were greater in the HF group than in the normal group, and apocynin significantly suppressed O₂⁻ production and NADPH oxidase activity compared to the HF group.
Figure 1: Left panel: Vascular superoxide levels in aortic tissues from the normal (open bars, n=5), the HF groups (closed bars, n=5) and the apocynin group (dotted bars, n=5). Right panel: NADPH oxidase activity in aortic segments from the normal (open bars, n=5), HF (closed bars, n=5), and apocynin groups (apocynin; dotted bars, n=5). *p<0.05 compared with the normal group, †p<0.05 compared with the HF group.

**Figure 2:** qRT-PCR analysis of selected genes involved in SOCS3, GADD45A, CDKN1A and DUSP5 in HF. Gene expression was normalized using the GAPDH gene in normal samples. Error bars represent standard errors from three experiments. †P<0.05 compared with the normal group, †P<0.05 compared with the vehicle group.

**Discussion**

Endothelial function is significantly attenuated and reduced skeletal muscle blood flow and correlates with the severity of symptoms in HF. Improvement of its dysfunction is an important target in the treatment of HF. The NADPH oxidase families are expressed in cardiovascular tissues and have been implicated in the control of vessel tone, inflammation, endothelial cell proliferation and migration [10]. In the present study, endothelium-dependent vasodilatation in dogs with tachycardia-induced HF was attenuated via an increase in the formation of vascular NADPH oxidase-derived O$_2^-$, accompanied by upregulation of the expression of SOCS3, GADD45A, CDKN1A and DUSP5. Apocynin (4-hydroxy-3-methoxy-acetophenone) is a methoxy-substituted catechol, was found to decrease ROS generation and we demonstrated that the agent inhibited the increased vascular NADPH stimulated O$_2^-$ production by nearly 68% of the baseline in HF, evaluated by lucigenin-enhanced chemiluminescence. Although apocynin did not change blood pressure and systolic cardiac dysfunction, it improved vascular dilated response to Ach.

To elucidate the molecular mechanisms that differentially control the expression, activation and cellular pathways linked to HF, as well as the physiological and pathophysiological contexts in which they exert their effects, are the important issues. We have reported approximately 15000 genes and identified changes in gene expression strongly related to occurrence of HF using global transcriptome analysis of NGS [5]. NGS revealed that expression of the p53 pathway-related genes and the inflammatory interleukin-related genes showed enhanced expression in HF compared with the normals. We selected the four core genes, SOCS3, GADD45A, CDKN1A and DUSP5, because expression of these genes overlapped within two transcriptome pathways. Apocynin suppressed activation of these genes compared to the HF group. SOCS3 encodes a member of the STAT-induced STAT inhibitor known as cytokine-inducible negative regulators of cytokine signaling. SOCS3 regulates important cellular processes such as proliferation and differentiation [11]. The expression is tightly controlled to avoid excessive inflammatory damage while maintaining effective control of pathogens. In SOCS3 deficient mice, tempol, a non-selective antioxidant, reversed angiotensin II-induced endothelial dysfunction by inhibiting IL-6 and STAT3 expression [12]. The GADD45 family (A, B and G) are implicated as stress sensors that modulate the response of mammalian cells to genotoxic/physiological stress. In addition to this role in cell cycle control, it mediates the activity of NF-kB in the cell death and survival control [13].

Although little is known about the role of GADD45 in the heart, Lucas et al. reported that GADD45G was significantly increased in the heart following myocardial infarction and overexpression of GADD45G induces cardiomyocyte apoptosis, fibrosis, left ventricular dysfunction, and HF. On the other hand, GADD45G in knockout mice confers resistance to ischemic injury, at least in part by limiting cardiomyocyte apoptosis [14]. The harmful effect of excessive ROS production has been linked to damage of macromolecules among which DNA damage is considered as the most relevant to the induction of senescence [15]. In human vascular endothelial cells, NADPH oxidase mediates serum starvation-induced cell cycle arrest by a mechanism that targets the expression of CDKN1A and p53. CDKN1A is a G1 checkpoint inhibitor leading to cell cycle arrest and the inhibition of DNA replication and CDKN1A is redox-sensitive functions as a regulator of cell cycle progression at G1 and S phase. [16].
DUSP5 is a serine-threonine phosphatase and dephosphorylates vascular endothelial growth factor-phosphorylated ERK1/2 inhibiting proliferation of endothelial cells [17]. Intrinsic vascular smooth muscle cell signaling in the cascade of events leading to augmented total peripheral resistance in HF and the active myogenic response regulates the local increase in microvascular resistance. DUSP5 also contributes to the impaired myogenic response which is an intrinsic property of vascular smooth muscle cell that enhances by the GADD45A, CDKN1A and DUSP5 genes expression in the aorta in HF.

Cytochrome at any given level of transmural pressure [18].

In the present study, we observed apocynin suppressed the SOCS3, GADD45A, CDKN1A and DUSP5 genes expression in the aorta in HF. Control of the vascular proliferation, senescence, inflammation and apoptosis could be contributed to the vascular protection by NADPH inhibition using apocynin against excessive ROS production in HF.

Conclusion

We can conclude that the specific inhibition of NADPH oxidase will become one of the promising therapeutic targets in the treatment of HF mediating through novel vascular molecular mechanisms.

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