Increased inactivation of nitric oxide is involved in coronary endothelial dysfunction in heart failure

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Arimura, Kenichi, Kensuke Egashira, Ryo Nakamura, Tomomi Ide, Hiroyuki Tsutsui, Hiroaki Shimokawa, and Akira Takeshita. Increased inactivation of nitric oxide is involved in coronary endothelial dysfunction in heart failure. Am J Physiol Heart Circ Physiol 280: H68–H75, 2001.—Recent evidence suggests the possibility that enhanced inactivation of endothelium-derived nitric oxide (NO) by oxygen free radical (OFR) may cause endothelial dysfunction in heart failure (HF). To test this hypothesis, we examined the effect of antioxidant therapy on endothelium-dependent vasodilation of the coronary circulation in a canine model of tachycardia-induced HF. Endothelium-dependent vasodilation was less than that in controls, and OFR formation in coronary arterial and myocardial tissues was greater in HF dogs than those in controls. The immunohistochemical staining of 4-hydroxy-2-nonenal, OFR-induced lipid peroxides was detected in coronary microvessels of HF dogs. Intracoronary infusion of the cell-permeable OFR scavenger Tiron inhibited OFR formation and improved endothelium-dependent vasodilation in HF dogs but not in controls. The NO synthesis inhibitor N^G-monomethyl-L-arginine (L-NMMA) diminished the beneficial effect of Tiron in HF dogs. Endothelium-independent vasodilation was similar between control and HF dogs, and no change in its response was noted by Tiron or Tiron plus L-NMMA in either group. In summary, antioxidant treatment with Tiron improved coronary vascular endothelium-dependent vasodilation by increasing NO activity in tachycardia-induced HF. Thus coronary endothelial dysfunction in HF may be, at least in part, due to increased inactivation of NO by OFR.

endothelium-derived factors; free radicals

HEART FAILURE (HF) is a serious health problem (6). In patients and animal models with HF, endothelium-dependent vasodilation has been demonstrated to be impaired in large arteries and microvessels of the coronary and peripheral circulation (4, 11, 16, 22, 25, 26, 28). Endothelial dysfunction in HF is likely to be caused mainly by reduced activity of nitric oxide (NO). Because of the importance of endothelial NO in coordinating tissue perfusion, the functional consequence of impaired activity of endothelial NO in the peripheral and coronary circulation in HF is, respectively, the reduction in exercise capacity and exacerbation of ventricular function. Regarding the pathogenesis of endothelial dysfunction in HF, Smith et al. (25) initially reported that gene expression of endothelial NO synthase and protein production of NO were reduced in a dog model of tachycardia-induced HF. Endothelial dysfunction in this setting can also be caused by impaired intracellular availability of L-arginine or an increased degradation of NO by increased production of oxygen free radicals (OFR). The latter possibility is supported by recent studies (2, 20) indicating the increase in OFR formation in HF. Hornig et al. (12) recently showed that, in patients with HF, the acute and chronic administration of the antioxidant vitamin C improves the endothelial function of conduit arteries by increasing the activity of NO. In the study by Hornig et al. (12), however, it is unclear whether vitamin C acted as an antioxidant or whether the beneficial effect of vitamin C in HF could be observed in the microcirculation. It is the small resistance vessels that coordinate tissue perfusion.

Therefore, the aim of the present study was to test the hypothesis that increased inactivation of endothelium-derived NO is involved in the mechanism of impaired endothelium-dependent dilation of the coronary circulation in a canine model of pacing-induced HF.

METHODS

Induction of Pacing-Induced HF

This study was approved by the Committee on the Ethics of Animal Experiments, Faculty of Medicine, Kyushu University, and was conducted according to the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University, and Law (No. 105) and Notification (No. 6) of the Japanese Government.

Experiments were performed in adult mongrel dogs (17–30 kg body wt). Under general anesthesia and fluoroscopic guidance, a bipolar pacing lead (1252T/58, Pace Setter, Sylmar, CA) was introduced into the right external jugular vein and advanced to the apical area of the right ventricle. The dogs were then allowed to recover from the surgery.

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HF was induced by rapid ventricular pacing at 240 beats/min for 4 wk (29). Ventricular pacing was performed continuously by connecting the lead to an external pulse generator (Nihon-Kohden, Tokyo, Japan). Control dogs were treated in identical manner as HF dogs without pacing.

On the day of the final study, all dogs underwent echocardiographic examinations in the conscious condition in sinus rhythm after 15 min of the stabilization period. Echocardiographic images were obtained with an ultrasonograph (SSH-160A, Toshiba Medical, Tokyo, Japan). Left ventricular (LV) short-axis (cross section) views were recorded at the papillary muscle level, and the internal LV dimensions and the thickness of the lateral wall of the LV were measured. The LV ejection fraction (in percent) was then calculated by the use of the following formula: 100 \times \left[\frac{\text{LV end-diastolic dimension} - \text{LV end-systolic dimension}}{\text{LV end-diastolic dimension}}\right]^3/2.

**Surgical Preparation**

After echocardiographic examination, we sedated the animals with intravenous diazepam (10 mg), intubated the animals, and ventilated the animals with a respirator. Dogs were then anesthetized with intravenous infusion of α-chloralose (3.75 mg/min) and urethane (37.5 mg/min). A thoracotomy was performed in the left fourth intercostal space, and the heart was suspended in a pericardial cradle. A heating pad was used to maintain the rectal temperature of animals within the range of 36.0 to 37.0°C.

A 7-Fr catheter was inserted into the aortic arch through the left carotid artery for measurement of aortic pressure (AoP), and a 7-Fr catheter-tip pressure transducer was inserted into the LV cavity through the left atrium for the measurement of LV pressure (LVP).

AoP was measured using a strain-gauge transducer (TP-400T, Nihon-Kohden). A cardiotachometer triggered by AoP pulses was used to monitor heart rate (HR). LVP was measured with a catheter-tip transducer (PC-350, Millar Instruments, Houston, TX), and the positive first derivative of LVP (LV dP/dt) was obtained by electronic differentiation. Coronary blood flow (CBF) was measured with an ultrasonic flowmeter (T201, Transonic System, Ithaca, NY). All variables were continuously monitored and recorded using a polygraph system (RM-6000, Nihon-Kohden).

A transit-time ultrasonic flow probe was placed at the midportion of the left anterior descending coronary artery (LADCA) (15). Peak responses of CBF to drugs were used for analysis. Heparin-filled tubing (2-Fr size) was inserted into the LADCA immediately distal to the flow probe for drug infusions. A 3-Fr catheter was inserted into the great cardiac vein and was advanced into the anterior interventricular coronary vein for venous blood sampling. The partial pressure of oxygen (P_{O_2}) and carbon dioxide (P_{CO_2}) and pH in arterial and coronary venous blood were measured with a gas analyzer (model 238, Chiron, Tokyo, Japan).

**Drugs**

Tiron (sodium dihydroxybenzene disulfonate), sodium nitroprusside (SNP), and N^6-monomethyl-L-arginine (L-NMMA) were obtained from Sigma (St. Louis, MO), and acetylcholine (ACh) was obtained from Dai-ichi (Tokyo, Japan). Tiron was dissolved in normal saline and neutralized by addition of equimolar NaOH. Other drugs were dissolved in normal saline.

**Experimental Protocols**

After completion of the surgical preparation, we studied the animals when all hemodynamic parameters were stabilized. The criteria for inclusion in the present experiments were 1) an Hb concentration > 9 g/dl; 2) an arterial pH of 7.35 to 7.45, a P_{O_2} of 100 to 200 mmHg, and a P_{CO_2} of 25 to 40 mmHg; and 3) coronary venous P_{O_2} < 30 mmHg.

**Protocol 1: effect of Tiron and Tiron plus L-NMMA on endothelium-dependent and endothelium-independent vasodilation.** Eight control dogs and eight HF dogs were used. The endothelium-dependent vasodilator ACh at graded doses (1, 3, and 10 μg/min) was infused into the LADCA while CBF at the LADCA and AoP, LVP, LV dP/dt, and HR were monitored continuously and recorded. After 5 min, all of the variables returned to baseline. The endothelium-independent vasodilator SNP (30 and 100 μg/min) was then administered. After the return to baseline, we infused Tiron at 7 mmol·1^{-1}·min^{-1} into the LADCA. Ten minutes after the beginning of Tiron infusion, the infusions of ACh and SNP were repeated during Tiron infusion.

After the subsequent return to baseline, we infused Tiron plus the NO synthase inhibitor L-NMMA (1 mg/kg) into the LADCA. ACh and SNP injections were repeated. We assumed Tiron to be a "cell-permeable antioxidant," because Tiron has been shown to scavenge OFR in both intracellular and extracellular environment (9, 17, 23).

**Protocol 2: reproducibility of vasodilatory responses to ACh and SNP.** Five control and five HF dogs were used. The ACh and SNP injection protocol was repeated three times at 30-min intervals without any treatment.

**Protocol 3: measurements of OFR formation.** Fourteen control dogs were used. The ACh and SNP injection protocol were repeated three times at 30-min intervals without any treatment. After the subsequent return to baseline, we infused Tiron plus the NO synthase inhibitor L-NMMA (1 mg/kg) into the LADCA. ACh and SNP injections were repeated. We assumed Tiron to be a "cell-permeable antioxidant," because Tiron has been shown to scavenge OFR in both intracellular and extracellular environment (9, 17, 23).

**Protocol 4: immunohistochemistry of 4-hydroxy-2-nonenal-modified protein.** To assess the cellular localization of lipid peroxidation by histochemical analysis, sections of LV myocardium were immunolabeled with an antibody raised against 4-hydroxy-2-nonenal (HNE)-modified protein, an aldehyde byproduct of lipid peroxidation (27, 30). Paraffin
embedded tissue sections (5 μm thick) were deparaffinized with xylene and reixed with Bouin’s solution for 20 min, immersed in PBS, and incubated with 0.3% hydrogen peroxide in methanol for 30 min. The sections were further incubated with polyclonal antiserum raised against a HNE-modified histidyl peptide (Gly3-His-Gly3) (4 or 8 μg/ml, NN2050–70, Funakoshi). After rinsing with 0.01 mol/l PBS, we incubated the sections with biotin-labeled goat anti-rabbit IgG antiserum (diluted 1:100; DAKO A/S) for 60 min and then incubated them with avidin-biotin complex (Vectastain ABC kit; 1:100) for 60 min. After rinsing, we finally incubated the sections with 0.02% 3,3-diaminobenzidine and 0.03% hydrogen peroxide in deionized water for 6 to 9 min. As a negative control, sections were incubated with normal rabbit serum as well.

Statistical Analysis

Data are presented as means ± SE. The differences between two experiments were compared using Student’s t-tests. The differences among three or more experiments were determined using two-way analysis of variance and a Bonferroni’s multiple comparison test. A P value of 0.05 or less was considered statistically significant.

RESULTS

Echocardiographic examination revealed that long-term pacing tachycardia caused a significant decrease in the LV ejection fraction (63 ± 3 and 27 ± 2%, respectively, in control and HF dogs) and an increase in LV end-diastolic dimensions (38 ± 1 and 46 ± 1 mm, respectively, in control and HF dogs). Hemodynamic parameters, which were measured under anesthesia, are shown in Table 1. Mean AoP and LV dP/dt were less (P < 0.01) and LVEDP was greater in HF dogs than in controls. There was no significant difference between the two groups in CBF and HR.

Effect of Tiron and Tiron Plus L-NMMA: Protocol 1

In control and HF dogs, treatment with Tiron or with Tiron plus L-NMMA did not affect basal CBF, other hemodynamic parameters, or myocardial metabolic states such as coronary venous Po2 and pH (Table 1).

The ACh-induced increase in CBF was significantly impaired in HF dogs than controls (Figs. 1 and 2). Tiron treatment significantly enhanced the ACh-induced increase in CBF in HF dogs but not in controls. SNP-induced increase in CBF was similar between the two groups, and no change in its response was noted by Tiron (Fig. 3). After Tiron treatment, the ACh-induced increase in CBF did not significantly differ between the two groups (Fig. 2).

Treatment with Tiron plus L-NMMA markedly reduced the ACh-induced increase in CBF in both control and HF dogs (Fig. 2). ACh-induced increases in CBF did not significantly differ between the two groups, and no change in its response noted by Tiron plus L-NMMA treatment (Fig. 3).

Time Control Study: Protocol 2

Changes in CBF in responses to ACh and SNP were not significantly different among the first, second, and third experiments (Table 2).

OFR Formation: Protocol 3

In dogs without in vivo Tiron treatment, the lucigenin chemiluminescence was used to assess OFR formation in the myocardium and coronary artery. ESFR spectroscopy was used to measure OFR formation in the myocardium. OFR formation in the coronary artery and myocardium was greater in HF dogs than in controls (Fig. 4). In dogs with in vivo Tiron treatment, OFR formation in the both tissues did not differ between the two groups.

Immunohistochemical Detection of Lipid Peroxidation: Protocol 4

Immunohistochemical analysis of HNE-modified protein was performed in four control dogs and four HF dogs. Lipid peroxides were positively stained in many coronary microvessels (small arteries, arterioles, and venules) in all four HF dogs without in vivo treatment with Tiron (Fig. 5). Large epicardial arteries and myocardial myocytes were weakly stained in HF dogs. Only weak stainings in the microvessels, large arteries, and myocardium were noted in HF dogs with in vivo Tiron treatment. In contrast, no labeling was observed in the

Table 1. Hemodynamic parameters before and after Tiron and after Tiron plus L-NMMA

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control dogs</th>
<th>Tiron plus L-NMMA</th>
<th>HF dogs</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Tiron</td>
<td>Tiron plus L-NMMA</td>
</tr>
<tr>
<td>CBF, ml/min</td>
<td>22 ± 4</td>
<td>23 ± 5</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>157 ± 1</td>
<td>158 ± 1</td>
<td>157 ± 2</td>
</tr>
<tr>
<td>Mean AoP, mmHg</td>
<td>84 ± 4</td>
<td>86 ± 5</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>6 ± 1</td>
<td>6 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>LV dP/dt, mmHg/s</td>
<td>1,956 ± 148</td>
<td>1,940 ± 160</td>
<td>2,014 ± 198</td>
</tr>
<tr>
<td>Artery</td>
<td>121 ± 12</td>
<td>120 ± 21</td>
<td>124 ± 18</td>
</tr>
<tr>
<td>Coronary vein</td>
<td>24 ± 3</td>
<td>22 ± 3</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>pH</td>
<td>7.33 ± 0.03</td>
<td>7.35 ± 0.03</td>
<td>7.33 ± 0.05</td>
</tr>
<tr>
<td>Artery</td>
<td>7.29 ± 0.02</td>
<td>7.33 ± 0.02</td>
<td>7.33 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 dogs in each group. CBF, coronary blood flow; HR, heart rate; AoP, aortic pressure; LVEDP, left ventricular (LV) end-diastolic pressure; LV dP/dt, peak positive derivative of LV pressure; l-NMMA, Nω-monomethyl-L-arginine. *P < 0.01, statistical significance versus corresponding control dogs.
coronary vessels or myocardium in control dogs. No immunoactivity was noted when the antibody against 4-HNE-modified protein was replaced with nonimmune IgG (negative control).

DISCUSSION

The major finding of the present study is that impaired endothelium-dependent, NO-mediated dilation of coronary circulation evoked by ACh in dogs with pacing-induced HF was improved by antioxidant treatment with Tiron. In contrast, the endothelium-dependent dilation of coronary circulation by ACh was not affected by Tiron in healthy control dogs. Furthermore, after Tiron plus 1-NMMA treatment, the ACh-induced dilation of coronary circulation was similar between the two groups. These findings suggest that the beneficial effect of Tiron observed in HF dogs was mediated by an increase in the bioactivity of endothelium-derived NO.

It is unlikely that Tiron nonspecifically increased the vasodilatory capacity of smooth muscle cells in the coronary vessels, because Tiron or Tiron plus 1-NMMA treatment did not affect the SNP-induced, endothelium-independent dilation in both control and HF dogs. We also considered the possibility that the beneficial effect of Tiron on ACh-induced dilation of coronary circulation attributed to changes in the severity of HF or in hemodynamic parameters. This possibility is
highly unlikely because intracoronary infusion of Tiron had no effect on hemodynamic parameters and the myocardial metabolic state (Table 1).

Several investigators have reported the increase in OFR formation in HF. Belch et al. (2) demonstrated that plasma lipid peroxides were increased in patients with HF compared with control subjects. Mallat et al. (20) measured pericardial fluid levels of 8-iso-prostaglandin F$_{2\alpha}$ (a specific maker of oxidative stress in vivo) and showed that pericardial levels of 8-iso-prostaglandin F$_{2\alpha}$ increased with the functional severity of HF and ventricular dilation. Mohazzab et al. (21) showed that superoxide anion production is increased in isolated failing human cardiac myocytes. In addition, cardiac antioxidant reserve may be reduced in experimental HF (10). In the present study, we have shown that OFR formation is increased in the coronary arterial and myocardial tissues of HF dogs compared with controls and that immunohistochemically demonstrable lipid peroxidation, induced possibly by increased OFR formation, can be detected in the vicinity of the coronary microvessels. In vivo treatment with Tiron did not affect the level of OFR formation in control dogs but did reduce it in HF dogs, suggesting that Tiron indeed acted as the antioxidant in our dogs with HF. The results of these prior studies and this study support the notion that OFR formation in cardiovascular tissue is increased in HF. Because OFR inactivates NO, the bioactivity of endothelium-derived NO would be impaired in HF, which in turn decreases endothelium-dependent vasodilation. Therefore, our present observation suggests that an increased inactivation of NO was involved in the impaired endothelium-dependent, NO-mediated dilation of the coronary circulation in the animal model of HF.

One caveat in interpreting our present observation involves a report that lucigenin itself can generate superoxide in the presence of cellular reductases in a cell-free system (5). However, whether significant autoxidation of lucigenin occurs in intact tissue is unclear. We measured OFR formation in the myocardial tissues with the use of ESR and detected increased OFR production in HF dogs. In addition, we tested the specificity of the lucigenin chemiluminescence and ESR spectroscopy with the use of the superoxide scavenger Tiron. These experiments suggest that autoxidation of lucigenin did not distort our data. Another caveat involves a fact that the magnitudes of HF are milder in the present study than those previously reported in terms of ACh response, LV function, systemic hemodynamics, and histopathological findings (14, 18, 25, 28). Kajstura et al. (14) reported that chronic ventricular pacing for 4 wk produced multiple foci of replacement fibrosis due to losses of myocytes comprising 6% of the LV wall. Such pathological changes were associated with inflammatory changes, such as mononuclear leukocyte infiltration and activation. However, such fibroinflammatory changes were less prominent in the present study (data not shown).

The mechanisms by which OFR formation are increased in HF were not explored in the present study. However, there are several potential causes. First,
there is evidence that plasma and tissue levels of angiotensin II are elevated in animals and humans with HF, including this model of HF (19). Angiotensin II has been shown to activate NADH/NADPH oxidase, which is thought to be the major enzyme responsible for increased OFR production in the blood vessel wall (7, 8). Other factors may be involved as well, such as infiltrating leukocytes into the heart and vessel (1) and increased levels of tumor necrosis factor-α.

Recent data suggest that the mechanism responsible for reduced endothelium-dependent vasodilation may be multifactorial (4). These multifactors include decreased gene expression and activity of endothelial NO synthase, possibly due to chronically reduced blood flow, increased endothelium-derived vasoconstricting factors, reduced availability of L-arginine, and increased OFR formation due to increased levels of cytokines (such as TNF-α), increased angiotensin II activ-

Fig. 4. Combined bar and dotted graphs for oxygen free radical formation. Oxygen free radical formation is assessed by lucigenin chemiluminescence (A) or by electron-spin resonance spectroscopy (B). Oxygen free radicals from the coronary artery or myocardium with and without in vivo Tiron treatment are presented. Open bars denote control dogs and hatched bars denote HF dogs. *P < 0.01 versus no treatment, †P < 0.01 versus corresponding control dogs.

Fig. 5. Immunohistochemical micrograph of the coronary microvessels (arterioles) stained for a 4-hydroxy-2-nonenal (HNE)-modified histidine peptide or nonimmune IgG. + and −, presence and absence of Tiron, respectively.
ity, and etc. (16). Our present observation demonstra-
ting the normalization by Tiron of endothelial NO-
mediated vascular vasodilation may support the hy-
pothesis that increased inactivation of endothelial NO
by increased OFR formation may be responsible for
endothelial dysfunction in HF.

In conclusion, the present study has demonstrated that
the antioxidant treatment with Tiron is capable of
improving and/or normalizing endothelial dys-
function of the coronary circulation in the dog model
of pacing-induced HF. Our present observations sug-
post that HF is associated with increased oxidative
stress, which in turn increases inactivation of NO
and thus impairs endothelial NO-mediated dilata-
tion of the coronary circulation. Our results extend a
previous study by Hornig et al. (12), which indicated
that the beneficial effect of vitamin C on endothelial dys-
function in large arteries in patients with HF. Chronic
HF is characterized by vasoconstriction and reduced perfusion of several organs, such as the
skeletal muscle, heart, and kidney. Thus, if the im-
provement of endothelial dysfunction by antioxidant
therapy results in amelioration of the reduced perfu-
sion of such organs, endothelial dysfunction of the
circulation may be a target for therapy to improve
ventricular function and exercise capacity in HF.
This claim needs to be confirmed in future studies.

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