β-Adrenergic receptors desensitization is not involved in exercise-induced cardiac fatigue: NADPH oxidase-induced oxidative stress as a new trigger

Damien Vitiello, Julien Boissière, Grégory Doucende, Sandrine Gayrard, Anne Polge, Patrice Faure, Aurélie Goux, Stéphane Tanguy, Philippe Obert, Cyril Reboul, and Stéphane Nottin

1Research Laboratory, Laboratory of Cardiovascular Adjustments to Exercise, Faculty of Sciences, University of Avignon; 2Research Laboratory, Physical Activity, Muscle, Health, Faculty of Sport Sciences and Physical Education, Lille-2 University, Ronchin; 3Biochemistry Laboratory, Hospital of Nîmes, Nîmes; 4Nutritional and Hormonal Biochemistry Laboratory, Hospital of Grenoble, Grenoble; 5Research Laboratory, INSERM U 1040, Faculty of Medicine and Pharmacy, Grenoble University, Grenoble; and 6Research Laboratory, Human Nutrition and Atherogenesis, University Institute of Clinical Research, Montpellier, France

Submitted 12 April 2011; accepted in final form 20 June 2011

Prolonged strenuous exercise (PSE), such as marathon or long-duration triathlon, induces transient left ventricle (LV) dysfunction. Previous studies suggest that β-adrenergic pathway desensitization could be involved in this phenomenon, but it remains to be confirmed. Moreover, other underlying mechanisms involving oxidative stress have been recently proposed. The present study aimed to evaluate the involvement of both the β-adrenergic pathway and NADPH oxidase (Nox) enzyme-induced oxidative stress in myocardial dysfunction in rats following PSE. Rats were divided into 4 groups: controls (Ctrl), 4-h exercised on treadmill (PSE), and 2 groups in which Nox enzyme was inhibited with apocynin treatment (Ctrl APO and PSE APO, respectively). We evaluated cardiac function in vivo and ex vivo during basal conditions and isoproterenol stress. GSH/GSSG ratio, cardiac troponin I (cTnI) release, and lipid peroxidation (MDA) were evaluated. PSE induced a decrease in LV developed pressure, intrinsic myocardial contractility, and relaxation associated with an increase in plasma cTnI release. Our in vivo and ex vivo results demonstrated no differences in myocardial response to isoproterenol and of effective dose 50 between control and PSE rats. Interestingly, the LV dysfunction was reversed by apocynin treatment. Moreover, apocynin prevented cellular oxidation [GSH/GSSG ratio: PSE APO rats vs. PSE rats in arbitrary units (au): 1.98 ± 0.07 vs. 1.35 ± 0.10; P < 0.001]. However, no differences in MDA were observed between groups. These data suggest that myocardial dysfunction observed after PSE was not due to β-adrenergic receptor desensitization but could be due to a signaling oxidative stress from the Nox enzyme.

ADDRESS FOR REPRINT REQUESTS AND OTHER CORRESPONDENCE: V. Damien, Laboratory of Cardiovascular Adjustments to Exercise, Univ. of Avignon, 33 Rue Louis Pasteur, 84000 Avignon, France (e-mail: damien.vitiello@univ-avignon.fr)

METHODS

Ethical Approval

All procedures were performed in agreement with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85–23, revised 1996) and approved by the French ministry of agriculture.
Animal Care

Adult male Wistar rats (250–325 g; 7–10 wk old; n = 112; Charles River Laboratories, Lyon, France) were used in this study. All animals were housed in a temperature-controlled facility, provided with standard rat chow and water ad libitum, and adapted to a 12:12-h light/dark cycle in a temperature of 21°C.

Adults and prolonged strenuous exercise. Adult male Wistar rats were randomly assigned to the following groups: a first set of rats were control rats (Ctrl) and a second set were exercised rats (PSE). All exercise sessions were performed on a motorized rodent treadmill. All animals were familiarized with the treadmill over 5 days. Control rats were placed on the treadmill to experience the same restraint as PSE rats. On the last day, PSE rats were submitted to an adapted incremental endurance test. Briefly, the test started with a 5-min warm-up at 7.5 m/min running speed. Running speed was then increased by 1.5 m/min every 2 min until rats could no longer maintain pace with the treadmill. Two days after the incremental test (to avoid the fatigue effect of the test), rats performed a prolonged bout of exercise for 4 h at 60–65% of the maximal running speed achieved during the incremental test. To avoid the effect of recovery, all animals were killed within 30 min following PSE for the in vivo and isolated heart experiments.

Implication of β-Adrenergic Pathway

In vivo hemodynamic investigations. After the run, a first set of rats (n = 20) were intraperitoneally anesthetized (60 mg/kg pentobarbital sodium). Evaluation of both arterial and ventricular pressures was subsequently performed in intact closed-chest rats. Measurements of left intraventricular pressure and its maximal rate of rise (dP/dt max) and decrease (dP/dt min) were carried out before and during the maximal response to β1/β2-adrenergic receptors agonists (isoproterenol, 1 mg·kg⁻¹·min⁻¹) with a Millar pressure-volume transducer. The analog outputs of the arterial pressure and heart rate were collected with an MP35 module (Biopac System, Goleta, Santa Barbara, CA). Data were processed with BIOPAC student Lab Pro 3.7 software.

Isolated heart investigations. In a second set of rats (n = 20), animals were anesthetized (60 mg/kg pentobarbital sodium) following the run and 50 U of sodium heparin was administered in the saphenous vein. The heart was then quickly removed and placed on a Krebs buffer (in mM: 118 NaCl, 5 KCl, 0.9 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 11 D-glucose, and 2.5 CaCl2 and equilibrated at 37°C) during the last 3 days before testing as previously described (28). Isolated heart investigations were made, as previously described, on four animal groups: Ctrl and PSE and Ctrl and PSE rats treated with apocynin.

Blood sampling. Collected samples (1.5 ml) were centrifuged (4,000 g, 10 min at 4°C) and stored at –80°C for further biochemical analyses of catecholamines and cardiac troponin I (cTnI), a marker of myocardial cell damage (ARCHITECT STAT Troponin-I Reagent Kit).

Malondialdehyde assay. Malondialdehyde (MDA) levels were determined in cardiac tissue as an indication of lipid peroxidation. Approximately 120 mg of frozen LV tissue were homogenized into 1 ml of 0°C cold HClO4 (7% vol/vol) solution with the Ultra-Turrax T25 Basic (Rose Scientific, Toronto, Canada) at 11,000 rpm for 30 s and then centrifuged at 3,000 rpm for 10 min at 0°C. The supernatant was incubated for 30 min at 100°C and centrifuged for 15 min at 3,000 rpm at ambient temperature. Finally, MDA tissue concentration was assessed with a fluorimeter (Spectronic Jenway 62 series, Garforth, G-B; excitation at 515 nm and emission at 535 nm).

Glutathione and glutathione disulfide assay. Glutathione levels were determined in cardiac tissue as an indication of oxidative stress. Frozen tissue samples were homogenized in four volumes (wt/vol) of 1% picric acid. After centrifugation (16,000 g, 30 min, 4°C), supernatant fractions were assayed for total glutathione and glutathione disulfide (GSH and GSSG) concentrations determination using the OxisResearch BIOXYTECH GSH/GSSG-412 kit. The procedure consisted of using linear regression of GSH and GSSG samples at 412 nm.

Statistical Analysis

Data were analyzed using one-way or two-way ANOVA among groups. When significant interactions were found, a Bonferroni t-test was applied with adjusted P < 0.05 (Statview 2.20; Adept Scientific, Letchworth, UK). Data are presented as means ± SE.

RESULTS

PSE-Induced LV Dysfunction

From in vivo evaluation (Fig. 1, A and B), we observed a decrease in cardiac function in PSE compared with Ctrl rats, characterized by a drop in LVDevP (Fig. 1B, left), dP/dt max (Fig. 1B, middle), and dP/dt min (Fig. 1B, right). This altered cardiac function is obvious in vivo in PSE rats despite higher plasma concentration of epinephrine (Fig. 1C, left) and norepinephrine (Fig. 1C, middle). Following such strenuous exercise, cardiac dysfunction is associated with increased plasma concentration of cTnI (Fig. 1C, right). Such cardiac dysfunction observed in vivo was also found ex vivo (Fig. 1D). Indeed, in the ex vivo testing under highly standardized conditions (loading conditions and circulating factors), a marked decrease in LVDevP (Fig. 1D, left), dP/dt max (Fig. 1D, middle), and dP/dt min (Fig. 1D, right) was observed in PSE rats compared with Ctrl rats.
Ctrl rats, hence highlighting an intrinsic myocardial dysfunction following prolonged strenuous exercise.

**Involvement of β-Adrenergic Pathway in Myocardial Dysfunction**

To evaluate the potential role of β-adrenergic receptor desensitization in PSE-induced myocardial dysfunction, we then evaluated cardiac responses to a β1/β2-adrenergic receptor agonist (isoproterenol) on an isolated perfused rat heart. The myocardial response to increasing doses of isoproterenol was identical in PSE rats compared with Ctrl rats. The delta of responses to isoproterenol infusion, calculated as the difference between maximal cardiac response to isoproterenol and function at baseline, revealed no difference in ΔLVDevP (Fig. 2A, left), ΔdP/dt max (Fig. 2A, middle), and ΔdP/dt min (Fig. 2A, right) between groups. In addition, according to normalized

---

**Fig. 1.** Left ventricular dysfunction after a prolonged strenuous exercise. A: representative recording of left ventricular developed pressure (LVDevP), LV contractility (dP/dt max), and LV relaxation (dP/dt min). LVDevP, dP/dt max, and dP/dt min after prolonged strenuous exercise (PSE) in vivo (B) and ex vivo (isolated perfused heart; D). Adrenaline, noradrenaline, and cardiac troponin I (cTnl) concentrations in plasma after PSE (C). Values are expressed ± SE. Significant differences between control (Ctrl) rats and exercised (PSE) rats *P < 0.05, **P < 0.001.
sensitivity curves, heart sensitivity (ED_{so}) to isoproterenol was not altered in PSE rats compared with Ctrl (Fig. 2B, right).

Involvement of Oxidative Stress in Myocardial Dysfunction

All results concerning involvement of oxidative stress in myocardial dysfunction are depicted in Table 1. To evaluate the potential role of oxidative stress and more particularly of NADPH oxidase activation by PSE, rats were treated with apocynin, a specific inhibitor of NADPH oxidase, prior to exercise test. PSE-induced oxidative stress is evidenced by the decrease in GSH/GSSH ratio (Fig. 3A, left), with increase cTnI release (Fig. 3A, middle) and no alteration of tissular MDA (Fig. 3A, right), a marker of lipid peroxidation. In PSE rats treated with apocynin, GSH/GSSH ratio was normalized (Fig. 3A, left) and cTnI release partially blunted (Fig. 3A, middle), thus suggesting a major role of NADPH oxidase in the aforementioned PSE-induced oxidative stress. The specific inhibition of NADPH oxidase during PSE improved myocardial function in PSE rats to the level of Ctrl rats (Fig. 3B). Similarly, LVDevP (Fig. 3B, left), dP/dt_{max} (Fig. 3B, middle), and dP/dt_{min} (Fig. 3B, right) were normalized in PSE rats to levels found in Ctrl rats.

DISCUSSION

The major findings of the present study indicate that 1) prolonged strenuous exercise induces impairment in intrinsic myocardial function, 2) β-adrenergic receptor desensitization is not involved in this phenomenon, and 3) NADPH oxidase-induced modification of redox status is a potential new trigger of PSE-induced cardiac dysfunction.

PSE-Induced Intrinsic Myocardial Dysfunction

In humans, long-duration exercise such as marathon or long-duration triathlon induces a decrease in LV function (2, 5,

---

Table 1. Myocardial dysfunction and Nox-dependent oxidative stress

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>Ctrl APO</th>
<th>PSE</th>
<th>PSE APO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nox-dependent oxidative stress</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH/GSSH ratio, au</td>
<td>2.15 ± 0.15</td>
<td>1.93 ± 0.09</td>
<td>1.35 ± 0.10^*</td>
<td>1.98 ± 0.07^*</td>
</tr>
<tr>
<td>cTnI, ng/ml</td>
<td>0.16 ± 0.04</td>
<td>0.19 ± 0.05</td>
<td>0.74 ± 0.06^*</td>
<td>0.55 ± 0.05^a</td>
</tr>
<tr>
<td>MDA, mmol/g</td>
<td>9.88 ± 1.00</td>
<td>7.54 ± 1.00</td>
<td>7.57 ± 1.00</td>
<td>7.40 ± 1.00</td>
</tr>
<tr>
<td><strong>Myocardial function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVDevP, mmHg</td>
<td>123 ± 7</td>
<td>115 ± 7</td>
<td>82 ± 2b</td>
<td>108 ± 4c</td>
</tr>
<tr>
<td>dP/dt_{max}, mmHg/s</td>
<td>4125 ± 200</td>
<td>3723 ± 157</td>
<td>2591 ± 93^a</td>
<td>3407 ± 134^d</td>
</tr>
<tr>
<td>dP/dt_{min}, mmHg/s</td>
<td>-2122 ± 74</td>
<td>-1829 ± 77</td>
<td>-1542 ± 78^*</td>
<td>-1923 ± 96^d</td>
</tr>
</tbody>
</table>

Values are means ± SE. cTnI, cardiac troponin I; dP/dt_{max}, index of contractility; dP/dt_{min}, index of relaxation; GSH/GSSH ratio, ratio of reduced glutathione to oxidized glutathione; LVDevP, left ventricular developed pressure; MDA, mMalondialdehyde. Significant differences between control (Ctrl) and prolonged strenuous exercise (PSE) rats: ^*P < 0.05; ^aP < 0.01; ^bP < 0.001 and between PSE and PSE apocynin(APO) rats: ^cP < 0.05; ^dP < 0.001.
In the present study, we used an animal model to obtain “exercise-induced cardiac fatigue.” The results obtained in vivo in the present study are comparable to those obtained in humans since PSE resulted in reduced cardiac function despite elevated circulating plasma catecholamines. The high level of circulating catecholamines accompanied by potential alterations of heart loading conditions are confounding factors that preclude any conclusion regarding intrinsic myocardial dysfunction after PSE. Therefore, using an isolated perfused rat heart model allowed us to avoid the influence of such confounding factors. A major result of the present study is that myocardial function after strenuous exercise is markedly impaired. This result strongly suggests, for the first time, that PSE results in intrinsic myocardial fatigue. This conclusion is in line with recent studies in humans using new advances in echocardiography, less influenced by loading conditions and heart rate (5, 11, 23).

Implication of β-Adrenergic Pathway in PSE-Induced LV Dysfunction

The underlying mechanisms responsible for this deterioration in myocardial function after PSE are subject to ongoing debate. Recent studies suggest that β-adrenergic receptor desensitization could be one potential mechanism (13, 27). However, results in the literature are inconclusive, with some studies reporting altered cardiac responses to β-adrenergic receptor agonists after PSE (2, 7), while others found no change (16). Such contradictions could be explained by PSE induced 1) persistent higher levels of circulating catecholamines, 2) changes in blood plasma volume, subsequently of heart loading conditions, and 3) altered vascular resistance. Therefore, a main finding of the present study is the absence of change in cardiac response to a β1/β2-adrenergic receptor agonist in the isolated PSE rat hearts. This result suggests that PSE is not associated with alteration of heart response to β-adrenergic receptor stimulation, and in our experimental model, PSE-induced in vivo cardiac dysfunction is not explained by alteration of the β-adrenergic receptor pathway.

Implication of NADPH Oxidase-Induced Oxidative Stress in PSE-Induced LV Dysfunction

Among the potential mechanisms, oxidative stress can be involved in PSE-induced LV dysfunction. As seen, in our model, an increase in oxidative stress was reported in myocardial tissue after PSE, characterized by redox status alterations. Despite oxidative stress being associated with cardiac injuries in PSE rats, characterized by increased plasma cTnI release, no detectable change is reported regarding lipid peroxidation. However, moderate oxidative stress is sufficient to alter myocardial function after PSE. NADPH oxidase is regarded as a main pathological source of superoxide anion in the myocardium (12, 18, 24). In addition, its activity is increased with exercise (25). In this study, Sanchez et al. (25) proposed that the activation of NADPH oxidase by a short-duration exercise...
EXERCISE-INDUCED MYOCARDIAL DYSFUNCTION

induces reactive oxygen species production leading to redox modification of the ryanodine receptor-2, which then participate in cardiac preconditioning response and cardioprotection. However, to date, the effect of NADPH oxidase activation by long-duration exercise on myocardial function has not been previously described. Therefore, a major result of the present study is that myocardial functional alterations, resulting from PSE, are normalized when NADPH oxidase is specifically inhibited during PSE, suggesting that NADPH oxidase is mainly implicated in PSE-induced myocardial dysfunction. This result is explained in our work by a reduction in oxidative stress in the presence of apocynin. Accordingly, NADPH oxidase inhibition during PSE normalized redox status and then partially blunted myocardial damages, as evidenced by reduction of plasma cTnI release in PSE-treated rats. This result strongly suggests that myocardial damage after PSE partially result from NADPH oxidase-induced oxidative stress as supposed by others (4). Cardiac oxidative stress is implicated in the impairment of cardiac function through oxidative damage to cellular proteins and membranes (14, 17). However, because cTnI release is only partially corrected, we hypothesize that the deleterious effects of oxidative stress are also mediated by other mechanisms. The action of oxidative stress in modulating the activity of diverse intracellular signaling pathways and molecules is well described in the literature. For example, key proteins involved in myocardial excitation-contraction coupling, such as ion channels, sarcoplasmic reticulum calcium release channels, and myofilament proteins can undergo redox-sensitive alterations during activity (30). Finally, the superoxide anion is a potent deactivator of the signaling molecule nitric oxide. In pro-oxidant conditions, the reduction in NO bioavailability could also contribute to vascular endothelial dysfunction and lead to the loss of other physiological effects of nitric oxide (29). However, in our model such alterations remain hypothetical and further studies are needed to better understand how oxidative stress from NADPH oxidase can induce LV dysfunction after PSE.

Conclusion

Prolonged strenuous exercise on a treadmill in rats led to in vivo cardiac dysfunction, similar to that observed in humans. In addition, our study is the first to show that prolonged strenuous exercise results in intrinsic myocardial dysfunction, independent of heart loading conditions, circulating factors, and β-adrenergic receptor desensitization. Finally, we found a significant link between NADPH oxidase-dependent oxidative stress and myocardial dysfunction after PSE, which represents a new trigger in the comprehension of PSE-induced cardiac fatigue.

Clinical Implications

From a clinical point of view, our findings indicated that LV dysfunction observed after long-duration exercise is not only a consequence of alteration in loading conditions but also the consequence of an intrinsic decrease in myocardial contractility and relaxation. The present study highlights the link between NADPH oxidase-induced oxidative stress and myocardial dysfunction associated with moderate myocardium damage (i.e., no increase in MDA myocardial tissue concentration) after PSE. Our results are indicative of a signaling oxidative stress, probably leading to transient vascular and/or cellular alterations, as observed in “myocardial stunning” (3). However, the long-term consequences of repetitive bouts of such prolonged strenuous exercise are unknown and represent an important issue for future investigations.

GRANTS

This research was supported by research funds from Sanofi-aventis and the French Society of Cardiology (SFC; Grant “Heart and Sports”).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


