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

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Exercise-induced oxidative stress has been proposed as another main contributor (15). Among sources of ROS production, NADPH oxidase is commonly identified in the cardiovascular system and well known for its role in cardiac dysfunctions (18). In addition, it is of interest to note that NADPH oxidase activity is increased in response to tachycardia or exercise (25). However, to the best of our knowledge, the potential role of NADPH oxidase-induced oxidative stress in myocardial dysfunction following PSE has never been challenged.

In this context, using an experimental model of PSE in rats, we aimed to evaluate 1) whether PSE could result in intrinsic myocardial dysfunction and 2) whether β-adrenergic receptor desensitization and/or NADPH oxidase-induced oxidative stress could be involved in such myocardial dysfunction.

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.

**Animals 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 subjected to retrograde perfusion under constant pressure, isolated perfused Langendorff heart model. The heart was then quickly removed and placed on a perfusion dish with 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 with 95% O2–5% CO2 at 37.5°C). An ultrathin water-filled balloon tied to a microtip catheter pressure transducer (MP35 SS13L, Gotela) was introduced into the left ventricle to normalize the diastolic blood pressure and to record cardiac function. The hearts were electrically paced at 300 beats/min with an electrical stimulator (low voltage stimulator, BSL MP35 SS58L, Gotela). Following a 20-min equilibration period, baseline values of left ventricular developed pressure (LVEDP), dP/dt\text{max} and dP/dt\text{min} were recorded using an MP 35 module (Biopac system, Gotela) and processed with BIOPAC student Lab Pro 3.7 software. The procedure consisted of using linear regression of GSH and glutathione disulfide concentration 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 62series, Garforth, G-B; excitation at 515 nm and emission at 553 nm).

**Implication of Oxidative Stress**

Isolated heart investigations. In the third set of rats (n = 40) Ctrl and PSE rats were treated with apocynin, a bio-absorbable (Sigma Aldrich, St. Quentin Fallavier, France) specific inhibitor of the NA-DPH oxidase enzyme. Apocynin was given in drinking water (1.5 mM) 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.

**Biochemical Assays**

After exercise, in a last set of rats (n = 32), venous blood samples were obtained and hearts were quickly removed, trimmed of surrounding connective tissue and fat, and the LV was isolated, frozen in nitrogen liquid, and stored at −80°C for further assays.

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 in 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 62series, Garforth, G-B; excitation at 515 nm and emission at 553 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 LVEDP (Fig. 1B, left), dP/dt\text{max} (Fig. 1B, middle), and dP/dt\text{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 LVEDP (Fig. 1D, left), dP/dt\text{max} (Fig. 1D, middle), and dP/dt\text{min} (Fig. 1D, right) was observed in PSE rats compared with...
Ctrl rats, hence highlighting an intrinsic myocardial dysfunction following prolonged strenuous exercise.

**Involvement of  $\beta$-Adrenergic Pathway in Myocardial Dysfunction**

To evaluate the potential role of $\beta$-adrenergic receptor desensitization in PSE-induced myocardial dysfunction, we then evaluated cardiac responses to a $\beta_1/\beta_2$-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 $\Delta$LVDevP (Fig. 2A, left), $\Delta$dP/dt$_{\text{max}}$ (Fig. 2A, middle), and $\Delta$dP/dt$_{\text{min}}$ (Fig. 2A, right) between groups. In addition, according to normalized

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**Fig. 1.** Left ventricular dysfunction after a prolonged strenuous exercise. A: representative recording of left ventricular developed pressure (LVDevP), LV contractility (dP/dt$_{\text{max}}$), and LV relaxation (dP/dt$_{\text{min}}$). LVDevP, dP/dt$_{\text{max}}$, and dP/dt$_{\text{min}}$ 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_{50}) 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/GSSG 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/GSSG 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).

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,
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
Reduces 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 the 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 cTnl release in PSE-treated rats. This result strongly suggests that myocardial damage after PSE partially results 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 cTnl 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.

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**DISCLOSURES**

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

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