Pretreatment with brain natriuretic peptide reduces skeletal muscle mitochondrial dysfunction and oxidative stress after ischemia-reperfusion

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The mechanisms implicated in IR pathophysiology are highly dependent on mitochondrial function, the main energy source of cells. Indeed, prolonged IR in skeletal muscle has been found to significantly impair mitochondrial respiratory chain complex activities (6, 44), reduce adenine diphosphate-activated respiratory activity (state 3), decrease inner membrane potential and basal respiration (3, 34), and increase cytosolic/mitochondrial calcium overload (10, 26). Before cell death, increased radical oxygen species (ROS) induce cell membrane alterations, edema (18), and microvascular lesions (41). Apoptosis, an energy-requiring process resulting in nuclear fragmentation and cytoplasmic condensation, contributes to cell death and is also partly activated by a mitochondrial pathway initiated during ischemia and subsequently amplified during reperfusion (15, 31). Mitochondria are the cornerstones of the IR injury process, as well as targets for protective strategies (6, 44). Thus mitochondrial K\textsubscript{ATP} (mK\textsubscript{ATP}) channel opening has been shown to participate in the beneficial effects of preconditioning on different organs, including skeletal muscles (9, 29).

B-type natriuretic peptide (BNP), a hormone mainly secreted by the heart, is generally increased in the presence of impaired hemodynamic states and inflammation (43), such as congestive heart failure and circulatory shock (33, 40). It has been successfully used to decrease cardiac filling pressure and/or increase glomerular filtration rate (13, 17, 52).

At the subcellular level, D’Souza et al. were the first to demonstrate that BNP/mK\textsubscript{ATP} channel signaling constitutes an important injury-limiting mechanism during myocardial ischemia (11). Therefore, modulating BNP level may be an interesting therapeutic approach in patients suffering from IR-induced skeletal muscle alterations. Accordingly, although there are no data on a potential protective effect of BNP on skeletal muscles submitted to IR, BNP is known to inhibit apoptotic DNA fragmentation and to prolong survival of serum-deprived pheochromocytoma-derived PC12 cells (14).

Unlike the myocardium, the role of BNP during IR-induced skeletal muscle injuries has never been previously studied, to our knowledge.

The aim of the present study was, therefore, to determine, for the first time, whether pretreatment with BNP might protect hindlimb skeletal muscle against IR injuries, thereby preserving mitochondrial respiratory chain complex activities and reducing apoptosis. We hypothesized that BNP protection possibly involves mK\textsubscript{ATP} channel opening and reduced ROS production. We additionally investigated

SKELETAL MUSCLE INSULTS, SUCH as ischemia followed by reperfusion (IR), are common to many disease states observed in the intensive care unit. Indeed, shock, crush syndrome, or surgical procedures are associated with a prolonged IR in skeletal muscles, which is related to multiorgan failure syndrome (2, 4, 5, 27, 28).

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whether BNP injected during ischemia also displays protective properties.

MATERIALS AND METHODS

Animals

This study conforms to the European institutional guidelines for ethical animal treatment and was approved by the Animal Care Committee of the University of Strasbourg (C.R.E.M.E.A.S. no. AL/04/08/05/09). Experiments were performed on 44 adult male Wistar rats (Depré, France) weighing ~ 250–350 g. Animals were housed in a neutral temperature environment (22 ± 2°C) on a 12:12-h photoperiod and were provided food and water ad libitum.

Surgical Preparation and Procedures

Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (55 mg/kg body wt) and tracheotomized with a 14-gauge cannula for mechanical ventilation (SAR 830/P Small Animal Ventilator, CWE, Ardmore, Pa) (ambient air, 50 ml/min respiratory rate). The adequacy of anesthesia was monitored by a periodic observation of mucous membrane color, adequate mechanical ventilation adaptation, and loss of pedal reflex; when necessary, additional intraperitoneal injections of pentobarbital sodium were required during the surgical procedure to maintain deep anesthesia. Heart rate was continuously monitored during the protocol in all animals with three transcutaneous electrodes (Power Lab/16 SP, CHART V.4.1.2 software).

Each rat was placed on an electric blanket to maintain body temperature at 37°C, which was monitored by a rectal probe throughout the experiment (Homeothermic Blanket Control Unit, Harvard Apparatus, Holliston, MA). As this procedure was performed entirely under general anesthesia from which the animal did not recover consciousness (i.e., “nonrecovery” procedure), animals were euthanized by pentobarbital sodium overdose at the end of experiment to ensure a minimum of pain, suffering, and distress.

Study Design

Thirty-eight rats were divided into four groups: the sham group (SH, n = 8) underwent 6 h of general anesthesia and surgical manipulation similar to that performed in the three other groups, with the exception of hindlimb ischemia, which was not induced. The IR group (n = 10) underwent 3 h of ischemia, induced by a rubber-band tourniquet applied on the right root of the hindlimb, followed by a reperfusion period of 2 h. BNP-IR: pharmacological preconditioning animals (n = 10) underwent the same IR protocol with the addition of two subcutaneous 5 μg/kg injections of brain natriuretic peptide (BNP), performed 60 and 30 min before IR. BNP-IR: animals (5 mg/kg, n = 10) underwent the same BNP-IR protocol, but with the addition of intraperitoneal administration of 5-hydroxydecanoic acid (5-HD) injected 10 min before the first subcutaneous BNP injection.

One portion was used immediately for studying mitochondrial respiration, while the remainder was frozen in isopentane cooled by liquid nitrogen and stored for subsequent analysis.

Study of Muscle Mitochondrial Respiration

Measuring oxygen consumption ex vivo in skinned fibers is a unique means to determine the functional oxidative capacity of the skeletal muscle, allowing the study of the entire mitochondrial population within its cellular environment. A schematic representation of the mitochondrial respiratory chain with specific substrates and inhibitors, as well as of oxygraph traces, is presented in Fig. 2.

Brevity, the mitochondrial respiration was studied in saponin-skinned fibers of the white gastrocnemius, as previously described (53). Fibers were separated and permeabilized in a bath of solution S containing 50 μg/ml saponin for 30 min at 4°C, under gentle shaking. Permeabilized fibers were then washed for 10 min, to remove saponin, and placed in a bath with the respiratory solution for two 5-min periods, to remove all phosphates. Finally, oxygen consumption was measured polarographically using a Clark-type electrode in a 3-ml oxygraphic cell (Strathkelvin Instruments, Glasgow, UK). Basal oxygen consumption (V₀), and maximal fiber respiration (Vmax) rates were measured at 22.1°C under continuous stirring in the presence of saturating amount of adenosine diphosphate as a phosphate acceptor.

The relative contributions of the respiratory chain complexes I, III, and IV to the global mitochondrial respiratory rates were also determined. When Vmax was recorded, electron flow went through complexes I, III, and IV. Complex I was blocked with antimycin (0.02 mM), and complex II was stimulated with succinate (25 mM) to investigate complex II, III, IV activities (VIII). Therefore, NADH/NAD+ tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5 mM) and ascorbate (0.5 mM) were added as an artificial electron donor to cytochrome c. Under these conditions, the activity of cytochrome-c oxidase (complex IV) was determined as an isolated step of the respiratory chain (VTPMD/asc).

Fibers were then harvested and dried for 15 min at 150°C. Respiration rates were expressed as micromoles of O2 per minute per gram dry weight.

Fig. 1. Experimental design. Sham: control animals (n = 8) underwent 6 h of general anesthesia and were sham operated. IR: ischemia-reperfusion animals (n = 10) underwent 3 h of ischemia induced by a rubber band tourniquet applied on the right root of the hindlimb, followed by a reperfusion period of 2 h. BNP-IR: pharmacological preconditioning animals (n = 10) underwent the same IR protocol with the addition of two subcutaneous 5 μg/kg injections of brain natriuretic peptide (BNP), performed 60 and 30 min before IR. 5HD-BNP-IR: animals (5 mg/kg, n = 10) underwent the same BNP-IR protocol, but with the addition of intraperitoneal administration of 5-hydroxydecanoic acid (5-HD) injected 10 min before the first subcutaneous BNP injection.
Dihydroethidium Staining

To detect the presence of ROS in skeletal muscles, 10-μm-thick serial sections were cut on a cryostat microtome and thaw-mounted onto glass slides. After air-drying, slides were incubated (30 min at 37°C) with 2.5 μmol/l dihydroethidium (DHE) in phosphate-buffered saline. DHE produces red fluorescence when oxidized to ethidium bromide by ROS, including superoxide anion (30). After staining, sections were rinsed, air dried, mounted in Vectashield (Vector Laboratories, Burlingame, CA), and coverslipped. They were examined under an epifluorescence microscope (Nikon Eclipse E800) with a ×20 epifluorescence objective, and the emission signal was recorded with a Zeiss 573–637 nm filter. Micrographs were analyzed with Adobe Photoshop 6.0 (USA).

Apoptosis Analysis by Caspase-3 Activity Assay

Frozen muscle samples (~0.1 g) were homogenized in 500 μl of extraction buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1.0 mM EDTA, and 0.0001% Triton X-100. Samples were then frozen overnight at −20°C. Samples were thawed and centrifuged at 12,000 g for 5 min. The assay was then carried out according to the kit protocol (EnzChek Caspase-3 Assay Kit, Molecular Probes, Invitrogen, Carlsbad, CA) (37, 48). Briefly, 50 μl of tissue homogenate were incubated with 50 μl of Z-DEV-AMC substrate at 37°C for 1 h. Fluorescence was measured with a Victor3 Wallac 1,420 multilabel counter (PerkinElmer) with excitation and emission wavelengths of 355 and 460 nm, respectively. A portion of tissue homogenate was incubated with the caspase-3 inhibitor Ac-DEVD-CHO, in which an inhibition curve was generated. The enzyme activity was calculated as the difference between the two curves. All data are expressed as means ± SE and analyzed using Prism database (GraphPad Prism 5, Graph Pad Software, San Diego, CA). A one-way ANOVA was used in all groups for mitochondrial respiratory function, ROS production, and apoptosis analysis, followed by Newman-Keuls post hoc test between groups for multiple comparisons. Intragroup mitochondrial respiratory function comparisons (IR vs. CL) were performed by using a paired t-test. A P value <0.05 was considered significant.

Table 1. Primer sequences used for quantitative real-time PCR amplification

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer 5’–3’</th>
<th>Reverse Primer 5’–3’</th>
</tr>
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<tbody>
<tr>
<td>Bax</td>
<td>GCTGAGACACTGAGACTTCTTC</td>
<td>GAGGACTCCAGACAAAGA</td>
</tr>
<tr>
<td>Bcl2</td>
<td>CGACTTGTGACAGAATGCTCA</td>
<td>CCTGAAGAGTTTCCCTGACCA</td>
</tr>
<tr>
<td>GCB</td>
<td>CGACATCTGACAGCTCCAGA</td>
<td>CTGCACAGTGGAGCCTGATT</td>
</tr>
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RESULTS

There were no differences between animals with regard to age, weight, and body temperature.

**IR Reduced Mitochondrial Respiratory Chain Complex Activities and Increases Bax-to-Bcl2 mRNA Ratio and Oxidative Stress**

IR decreased mitochondrial respiratory chain complex activities in the ischemic leg. See Fig. 3. In the glycolytic gastrocnemius muscle, maximal oxidative capacities (V\(\text{max}\)) reflecting complex I, III, IV activities were significantly impaired after IR, compared with the SH group (−36%, 3.7 ± 0.3 vs. 5.8 ± 0.2 \(\mu\)mol O\(_2\)·min\(^{-1}\)·g dry wt\(^{-1}\), \(P < 0.01\)). Regarding complexes II, III, IV, we observed a nonsignificant impairment of their activities after IR compared with the SH group (-23%, 3.3 ± 0.3 vs. 4.3 ± 0.3 \(\mu\)mol O\(_2\)·min\(^{-1}\)·g dry wt\(^{-1}\), \(P > 0.05\)). Finally, \(V_{\text{TMPD/asc}}\), reflecting complex IV activity, was significantly decreased after IR compared with the SH group (-37%, 8.6 ± 0.8 vs. 13.7 ± 0.9 \(\mu\)mol O\(_2\)·min\(^{-1}\)·g dry wt\(^{-1}\), \(P < 0.01\)).

IR likely increased apoptosis. See Fig. 4. IR likely increased apoptosis, as reflected by a significantly higher gene expression of Bax-to-Bcl2 mRNA ratio (Bax/Bcl2 mRNA) compared with the SH group (+57%, 1.1 ± 0.1 vs. 0.7 ± 0.1, \(P < 0.05\)).

IR also increased oxidative stress. See Fig. 5. To examine the implication of oxidative stress, DHE staining was used as an approach for measuring ROS production (and particularly superoxide anion). In gastrocnemius muscles, IR tended to increase the level of fluorescence compared with the SH group (+45%, 9.067 ± 935 vs. 6.249 ± 723 pixels, \(P > 0.05\)).

**Pretreatment with BNP Protected Skeletal Muscles Against IR**

BNP administered before ischemia protected mitochondrial respiratory chain complex activities. See Fig. 3. V\(\text{max}\), V\(\text{succ}\), and \(V_{\text{TMPD/asc}}\) were significantly higher in BNP-IR than in the IR group (+38%, 5.1 ± 0.5 vs. 3.7 ± 0.3 \(\mu\)mol O\(_2\)·min\(^{-1}\)·g dry wt\(^{-1}\), \(P < 0.05\); +67%, 5.5 ± 0.5 vs. 3.3 ± 0.3 \(\mu\)mol O\(_2\)·min\(^{-1}\)·g dry wt\(^{-1}\), \(P < 0.001\); +74%, 15.0 ± 1.3 vs. 8.6 ± 0.8 \(\mu\)mol O\(_2\)·min\(^{-1}\)·g dry wt\(^{-1}\), \(P < 0.001\), respectively).

Fig. 3. Mitochondrial respiratory chain complex I, II, III, and IV activities. Data are presented in four experimental conditions: sham (SH), after IR, BNP preconditioning (BNP-IR), and antagonized preconditioning (5HD-BNP-IR) groups. Results are means ± SE. **\(P < 0.01\) vs. SH, \(P < 0.05\) vs. BNP-IR. §§§\(P < 0.001\) (middle) and §§\(P < 0.01\) (bottom) vs. BNP-IR.

Fig. 4. Skeletal muscle apoptosis level: mRNA expression level ratio of Bax and Bcl2 detected by quantitative RT-PCR. Data are presented in four experimental conditions: SH, after IR, BNP-IR, and 5HD-BNP-IR groups. Results are means ± SE. *\(P < 0.05\) vs. SH. §§§\(P < 0.01\) vs. BNP-IR.

Fig. 5. Skeletal muscle oxidative stress. A: representative muscle dihydroethidium staining. Micrographs are of skeletal muscle in SH, IR, BNP-IR, and 5HD-BNP-IR groups. B: intensity of fluorescence. Data are presented in four experimental conditions: SH, after IR, BNP-IR, and 5HD-BNP-IR groups. Results are means ± SE. §§\(P < 0.01\) vs. BNP-IR.

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view text
IR Impairs Mitochondrial Respiratory Chain Complex Activities, Increases ROS Production, and Likely Increases Apoptosis

Measurements of mitochondrial function allow assessment of functional metabolic impairments of skeletal muscle, and the respiratory rate in skinned fibers is a unique means to measure the functional oxidative capacity of the entire mitochondrial population within its cellular environment.

Confirming previous data (44, 45), this study demonstrates that 3-h ischemia followed by 2-h reperfusion significantly decreased mitochondrial function in the glycolytic gastrocnemius muscle, a muscle readily mimicking the metabolic characteristics of deconditioned human skeletal muscle (3). On further exploring the functional activity of the different complexes, we observed a global impairment of the mitochondrial respiratory chain. Several mechanisms may contribute to these IR-induced deleterious effects, although increased oxidative stress appears to be a key factor (6). Indeed, the mitochondrial respiratory chain is one of the main sources of endogenous ROS, while the electron transport chain, which is central to the oxidative production of ATP in cells, has been shown to play an important role in oxidative stress injury. In particular, mitochondrial complex inhibition is known to increase ROS generation (7). Such increase in ROS production, in turn, exacerbates mitochondrial dysfunction and further induces oxidative damage to lipids and proteins, leading to alterations in major cell constituents and apoptosis (10, 18, 21, 36).

Apoptosis is widely recognized as an important cause of cell death after reperfusion in the myocardium (12), whereas much less and often controversial data are available in skeletal muscle (8, 23, 20, 49). Our results support the notion that IR induces an apoptotic process in skeletal muscle, as inferred from the greater Bax/Bcl2 mRNA in ischemic vs. sham-operated muscles. However, caspase-3 activity failed to change significantly in the four groups studied. Caspase-3 activity is considered as a good marker of apoptosis, and thus we expected an increase after IR and normalization after BNP pretreatment. Our study design may be at the basis of this discrepancy. Indeed, while the increase in Bax/Bcl2 mRNA occurs early in the setting of IR, caspase-3 is involved later in the apoptotic cascade. Thus 2 h of reperfusion may have been too short to quantify changes in caspase-3. Accordingly, in a canine model of myocardial IR subjected to 60 min of left anterior descending coronary occlusion, Zhao et al. observed a progressive occurrence of apoptosis from 6 to 72 h of reperfusion through detection of terminal transferase-mediated dUTP nick end-labeling-positive cells (51). Moreover, Tran et al. (47) recently reported tourniquet-induced apoptosis using caspase-3 activity measurement in mouse skeletal muscle following 3-h ischemia and 4-h reperfusion. Therefore, although requiring confirmation, we believe that apoptosis is involved in the deleterious effects of IR on skeletal muscle.

Pretreatment with BNP Protects Mitochondrial Respiratory Chain Complex Activities and Likely Prevents Skeletal Muscle Apoptosis

In this study, we demonstrate that BNP protects the mitochondrial respiratory function of skeletal muscle from IR injuries. BNP also likely prevents apoptosis. Indeed, mitochondrial respiratory chain complex activities were restored, and BNP decreased proapoptotic Bax mRNA gene expression while increasing antiapoptotic Bcl2 mRNA gene expression, thereby decreasing the Bax/Bcl2.

Ischemic pre- or postconditioning has been shown to protect muscle mitochondrial function against IR-induced deleterious effects (6, 44). However, pharmacological conditioning is easier to perform in a clinical setting. Unlike renin-angiotensin-aldosterone inhibitors, which have been shown to be ineffective (46), BNP appears as a good candidate. Indeed, in addition to the protection demonstrated in this study, BNP also displays beneficial renal and cardiovascular effects in the setting of cardiac surgery and transplantation (13, 16, 17, 52).

With regard to apoptosis, our results are in agreement with the data of Fiscus et al. (14), showing an antiapoptotic effect of BNP in PC12 cells. Similarly, others have also highlighted the antiapoptotic properties of BNP on cardiomyocytes in culture submitted to IR (42).

Knowing that ROS largely participates in the deleterious effects of IR and that BNP reduces the extent of myocardial infarction after coronary occlusion through mKATP channel opening (11), we investigated whether similar mechanisms could participate in the protection afforded by BNP on skeletal muscles.

Involvement of ROS and mKATP Channels in the Protective Effects of BNP

Lower DHE staining in IR skeletal muscle pretreated with BNP strongly supports the role of this cardiac hormone in decreasing oxidative stress. Since BNP herein reduced IR-induced mitochondrial dysfunction, the resulting reduced oxidative stress could be related, at least in part, to a better functioning of the mitochondrial respiratory chain, thereby reducing ROS overproduction (54). Consistently, cardiomyocytes in culture submitted to IR (42), as well as cardiac fibroblasts of newborn rats, have demonstrated a beneficial dose-dependent effect of natriuretic peptide against ROS overproduction (24).

As in the myocardium (11), mKATP channels may be involved in the protective effect of BNP against the deleterious effects of IR on skeletal muscle. Indeed, when 5-HD was administered in rats before BNP, impairments in mitochondrial respiratory chain complex activities were observed, as were increased apoptosis and ROS overproduction. Accordingly, in L6 skeletal muscle myoblasts and rat skeletal muscle mitochondria, potassium channel opener modulation of oxidative phosphorylation was abolished by glibenclamide, a well-known nonselective KATP channel inhibitor (9). In C2C12 myotubes, in the setting of preconditioning-induced cytoprotection, mitochondrial-enhanced oxygen consumption was attenuated by 5-HD (29). This cytoprotection is seemingly attributed to mKATP channels, since diazoxide, via opening of specific mKATP channels, was found to preserve mitochondrial respiratory function, reduces the output of ROS, as well as displays antiapoptotic properties (39), all of which were abolished by 5-HD (1). Diazoxide was also found to display similar effects in rat skeletal myoblasts, improving cell survival under oxidant stress in vitro, and in infarcted rat heart transplanted with myoblasts in vivo (32). Although the selectivity of 5-HD for mKATP channels remains a matter of debate and that 5-HD may also act on other intracellular targets (19), we cannot rule
out the cytoprotective effect of BNP on skeletal muscle, as least in part, by favoring mKATP channel opening.

Study Limitations

Despite adding caspase-3 activities to Bax/Bcl2 mRNA measurements, the apoptosis pathway has not been fully assessed in this work. The lack of change in caspase-3 may be related to the relatively short period of reperfusion and longer period of reperfusion might have been useful. Further analysis of caspase-9, which is involved earlier in the apoptotic cascade, may be preferable in this instance.

Moreover, we were able to show that BNP protects mitochondrial respiratory chain complex activities when injected before ischemia, but not during the ischemic episode. This could limit the therapeutic potential of BNP after insult, and BNP is thus more likely to act as a pharmacological preconditioner.

Moreover, it would be of interest to identify the signaling pathway leading BNP to mKATP channels opening, including the nature of the protein kinases involved and the presence of a potential, albeit still unknown, BNP receptor in skeletal muscle. A cellular approach may prove valuable in this context.

Perspectives

The results of our experimental study showing protective effects of BNP on IR-induced mitochondrial respiratory complex damage, ROS production, and proapoptotic effect are encouraging in view of its relevance in humans. Indeed, human skeletal muscle impairments are frequent, and, while sometimes subtle, they significantly participate in patient morbidity and mortality (27, 28). In human skeletal muscles, acute and chronic ischemias have been shown to induce mitochondrial dysfunctions (3, 35).

Although BNP failed to be protective when administrated during ischemia, exogenous BNP administration could prove useful in protecting muscle function in the setting of planned and relatively long periods of ischemia followed by reperfusion. This is observed in frequent surgical procedures, such as aortic aneurysm repair and reconstructive surgery with flap tissue transfer (22).

Conclusions

This study demonstrates, for the first time, in skeletal muscle that BNP is able to prevent IR-induced injuries, such as impaired mitochondrial respiratory chain complex activities, ROS production, and apoptosis. Furthermore, this protective effect of BNP on IR-induced injuries possibly involves mKATP channel opening. Since even subtle muscle impairments are associated with increased morbidity and mortality, further clinical studies are warranted to determine the clinical usefulness of BNP in the many settings of muscle IR, including crush syndrome, complex vascular surgery, or muscle flap procedures.

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Disclosures

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Author Contributions


References

18. Grisotto PC, dos Santos AC, Coutinho-Netto J, Cherri J, Piccinato CE. Indicators of oxidative injury and alterations of the cell membrane in...


