Myocardial ischemic postconditioning against ischemia-reperfusion is impaired in ob/ob mice

Omar Bouhidel,1,2 Sandrine Pons,1,2 Richard Souktani,2 Roland Zini,1,2 Alain Berdeaux,1,2,3 and Bijan Ghahle1,2,3

1Institut National de la Santé et de la Recherche Médicale U841, Équipe 3, 2Laboratoire de Pharmacologie, Faculté de Médecine, Université Paris 12, 3Groupe Henri Mondor-Albert Chenevier, Fédération de Cardiologie, and 4Plateforme Petit Animal, Institut Mondor de Médecine Moléculaire (IFR 10), Université Paris 12, Créteil, France

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Bouhidel O, Pons S, Souktani R, Zini R, Berdeaux A, Ghahle B. Myocardial ischemic postconditioning against ischemia-reperfusion is impaired in ob/ob mice. Am J Physiol Heart Circ Physiol 295: H1580–H1586, 2008. First published August 8, 2008; doi:10.1152/ajpheart.00379.2008.—Ischemic postconditioning (IPCD) significantly reduces infarct size in healthy animals and protects the human heart. Because obesity is a major risk factor of cardiovascular diseases, the effects of IPCD were investigated in 8- to 10-wk-old leptin-deficient obese (ob/ob) mice and compared with wild-type C57BL/6J (WT) mice. All animals underwent 30 min of coronary artery occlusion followed by 24 h of reperfusion associated or not with IPCD (6 cycles of 10-s occlusion, 10-s reperfusion). Additional mice were killed at 10 min of reperfusion for Western blotting. IPCD reduced infarct size by 58% in WT mice (33 mice were killed at 10 min of reperfusion. IPCD with IPCD (6 cycles of 10-s occlusion, 10-s reperfusion). Additional C57BL/6J (WT) mice. All animals underwent 30 min of coronary ischemia was confirmed by visualization of hyperemic cyanosis. Reperfusion was confirmed by visualization of hyperemic cyanosis.

The majority of these studies investigating postconditioning have been conducted in healthy animals, and there is a paucity of experimental investigations with associated risk factors such as hyperlipidemia, diabetes, or obesity. Importantly, these pathological situations as well as aging can jeopardize the effectiveness of well-established cardioprotective strategies in healthy animals (3, 19, 22, 37). To date, obesity is a major health issue in the Western countries, and its importance is constantly growing. This pathology constitutes a major risk factor for myocardial infarction and cardiovascular disease (16). Therefore, the effect of any developed cardioprotective strategy should be investigated in the context of this comorbidity.

Accordingly, the aim of the present study was to investigate the effects of postconditioning against myocardial infarction and subsequent activation of the RISK pathway in a mouse model of obesity. For this purpose, we chose the leptin-deficient ob/ob mouse, characterized by hyperphagia, decreased energy expenditure, and early onset of obesity (1).

METHODS

Animals. Male 8- to 10-wk-old wild-type C57BL/6J (WT) and ob/ob mice were used (R. Janvier, Le Genest St Isle, France). Mice were housed in an air-conditioned room with a 12:12-h light-dark cycle and received standard rodent chow and drinking water ad libitum. The authors have been granted a license from the institutional office “Préfecture du Val de Marne” (France) to conduct animal research.

Plasma measurements. Plasma glucose, triglyceride, cholesterol, and nonesterified fatty acid (NEFA) levels were measured with a multiparametric automat (Olympus automat type AU-400, Rungis, France).

Experimental protocol. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg), intubated, and ventilated mechanically. Body temperature was maintained at 37°C. A left thoracotomy was performed in order to realize sequences of coronary artery occlusion (CAO) followed by reperfusion (CAR) with an 8-0 Prolene thread placed around the left coronary artery as previously described (28). Myocardial ischemia was confirmed by ST segment deviation of the electrocardiogram and the occurrence of regional cyanosis. Reperfusion was confirmed by visualization of hyperemic response, and the chest was closed in layers.

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The experimental protocol is shown in Fig. 1. Both WT and ob/ob mice were randomly subjected to 30 min CAO (Control-WT and Control-ob/ob, respectively) or 30 min CAO followed by six cycles of 10-s CAR and 10-s CAO (IPCD-WT and IPCD-ob/ob, respectively). Infarct sizes were measured at 24 h of CAR. Additional animals underwent the same protocol but were killed at 10 min of CAR in order to perform Western blot experiments. Finally, hearts were also obtained from WT and ob/ob mice that did not undergo any intervention in order to perform Western blot analysis at baseline.

**Myocardial infarct size determination.** At the end of the 24-h reperfusion period, mice were reanesthetized, the coronary artery was reoccluded at the previous site of occlusion, and the heart was excised after Evans blue perfusion. The area at risk was identified by Evans blue staining, and the infarct area was identified by 2,3,5-triphenyl-tetrazolium chloride (TTC) staining. The area at risk was identified as the nonblue region and expressed as a percentage of the left ventricle weight. The infarcted area was identified as the TTC-negative zone and expressed as a percentage of the left ventricle weight. The infarct area was identified by 2,3,5-triphenyl-tetrazolium chloride (TTC) staining. The area at risk was identified as the nonblue region and expressed as a percentage of the left ventricle weight. The infarcted area was identified as the TTC-negative zone and expressed as a percentage of the left ventricle weight.

**Western blot analysis.** Extracted protein samples were denatured at 95°C for 5 min. Proteins (30 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with primary antibodies for phospho-Akt (serine 473), phospho-ERK1/2 (threonine 202/tyrosine 204), phospho-p70S6K1 (threonine 389), phospho-p70S6K1 (threonine 421/serine 424), phospho-AMP-activated protein kinase (AMPK) (threonine 172), total Akt, total ERK1/2, total p70S6K1, total AMPK, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (all from Cell Signaling Technology, Danvers, MA), MAP kinase phosphatase (MKP)-3 (Epitomics, Burlingame, CA), and protein phosphatase (PP2C (Abcam, Cambridge, UK). Blots were washed and then incubated with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were finally incubated with an enhanced chemiluminescence (ECL) detection system (ECL Western Blotting Substrate, Pierce) and exposed to ECL chemiluminescence film (Hyperfilm ECL, Ams-\*\*\*\*harn, Little Chalfont, UK). Bands of interest were scanned and quantified in a blinded manner with gel analysis software Image J-1.37 (National Institutes of Health, Bethesda, MD).

**Statistical analysis.** All values are expressed as means ± SE. Comparisons were performed with Kruskal-Wallis analysis followed by Mann-Whitney test. Statistical significance was defined as a value of P < 0.05.

**RESULTS**

**Biological and morphological parameters.** The biological characteristics of WT and ob/ob mice are summarized in Table 1. Serum glucose and cholesterol levels were significantly higher in ob/ob compared with WT mice. Similar concentrations of triglycerides and NEFA were observed in the serum of both strains.

As shown in Table 2, body weight was significantly increased by 1.9-fold in ob/ob compared with WT mice. Left ventricular weights were modestly (+13%) but significantly greater in ob/ob than in WT mice, and this mild left ventricular hypertrophy was confirmed by calculation of the left ventricular weight-to-tibia length ratio (also +13%).

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**Table 1. Glycemia, triglyceridemia, cholesterolemia, and NEFA measurements in WT and ob/ob mice**

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 6)</th>
<th>ob/ob (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemia</td>
<td>12.17 ± 0.63</td>
<td>24.69 ± 2.46*</td>
</tr>
<tr>
<td>Triglyceridemia</td>
<td>0.89 ± 0.07</td>
<td>0.68 ± 0.07</td>
</tr>
<tr>
<td>Cholesterolemia</td>
<td>2.78 ± 0.12</td>
<td>4.81 ± 0.17*</td>
</tr>
<tr>
<td>NEFA</td>
<td>1.28 ± 0.12</td>
<td>1.18 ± 0.08</td>
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Values (in mM) are expressed as means ± SE for n mice. WT, wild type; NEFA, nonesterified fatty acids. *P < 0.05 vs. WT.

**Table 2. General parameters in WT and ob/ob mice**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ob/ob</th>
</tr>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>25.5 ± 0.7</td>
<td>48.2 ± 1.2*</td>
</tr>
<tr>
<td>Left ventricular weight, mg</td>
<td>80.8 ± 2.1</td>
<td>88.2 ± 2.6*</td>
</tr>
<tr>
<td>Tibial length, mm</td>
<td>17.6 ± 0.1</td>
<td>17.3 ± 0.2</td>
</tr>
<tr>
<td>Left ventricular weight/tibial length, mg/mm</td>
<td>4.6 ± 0.1</td>
<td>5.1 ± 0.2*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE for n mice. IPCD, ischemic postconditioning. *P < 0.05 vs. respective WT.
Myocardial infarct sizes. The areas at risk were similar among all groups except for the ob/ob mice subjected to postconditioning [44 ± 3% (n = 9), 40 ± 3% (n = 9), and 41 ± 4% (n = 8) for Control-WT, IPCD-WT, and Control-ob/ob, respectively, compared with 27 ± 2% (n = 7) for IPCD-ob/ob]. The area at risk of postconditioned ob/ob mice was intentionally reduced. Indeed, in preliminary experiments, all ob/ob mice subjected to postconditioning with an area at risk averaging 40% died during reperfusion.

As illustrated in Fig. 2, postconditioning significantly reduced infarct size by 58% in WT mice (33 ± 1% vs. 14 ± 3% for Control-WT and IPCD-WT, respectively). In contrast, this strategy failed to protect ob/ob mice against myocardial infarction (53 ± 4% vs. 56 ± 5% for Control-ob/ob and IPCD-ob/ob, respectively). Interestingly, under control conditions, infarct size was significantly increased by 61% in ob/ob compared with WT mice.

Baseline analysis. As illustrated in Fig. 3, phospho-Akt(Ser473)-to-Akt, phospho-ERK1/2(Thr202/Tyr204)-to-ERK1/2, and phospho-AMPK(Thr172)-to-AMPK ratios were significantly increased in ob/ob (n = 5) compared with WT (n = 5) mice at baseline, i.e., in the absence of ischemia-reperfusion. Concomitantly, PTEN, MKP-3, and PP2C were significantly decreased in ob/ob compared with WT mice.

Effect of IPCD on PTEN, MKP-3, PP2C, and phosphorylation of Akt, ERK1/2, p70S6K1, and AMPK. The effects of IPCD on the phosphorylation states of prosurvival kinases Akt, ERK1/2, and p70S6K1 as well as AMPK were investigated in Control-WT (n = 3), IPCD-WT (n = 3), Control-ob/ob (n = 4), and IPCD-ob/ob (n = 4) mice.

Fig. 3. Western blot analysis of Akt and its phosphorylated (P) form at Ser473 (A), ERK1/2 and its phosphorylated forms at Thr202 and Tyr204 (B), p70S6K1 and its phosphorylated form at Thr389 due to the effect of Akt (C), p70S6K1 and its phosphorylated form at Thr421 and Ser424 due to the effects of ERK1/2 (D), AMP-activated protein kinase (AMPK) and its phosphorylated form (E), phosphatase and tensin homolog deleted on chromosome 10 (PTEN; F), MAP kinase phosphatase (MKP)-3 (G), and protein phosphatase (PP)2C (H) in WT and ob/ob mice at baseline (i.e., in the absence of ischemia-reperfusion). Values are means ± SE (n = 5/group for WT mice and 5/group for ob/ob mice). *P < 0.05 vs. WT.
As illustrated in Fig. 4A, IPCD induced a significant increase in the phospho-Akt(Ser473)-to-Akt ratio (+77%) in WT mice. Similarly, in WT mice, IPCD significantly increased the phosphorylated ERK1/2(Thr202/Tyr204)-to-ERK1/2 ratio (+41%) (Fig. 4B). In contrast, these effects were not observed in ob/ob mice. Under control conditions (i.e., in the absence of IPCD), these ratios were significantly greater in ob/ob compared with WT mice.

Because p70S6K1 is known to be phosphorylated at threonine 389 by activated Akt as well as at both threonine 421 and serine 424 by activated ERK1/2, we examined these phosphorylations. As illustrated in Fig. 5, IPCD induced significant increases in phosphorylated p70S6K1(Thr389)-to-p70S6K1 (+153%) and phosphorylated p70S6K1(Thr421/Ser424)-to-p70S6K1 ratios (+57%) in WT mice. This strategy failed to elicit similar effects in ob/ob mice.

As illustrated in Fig. 6, the phosphorylated AMPK(Thr172)-to-AMPK ratio was significantly increased by IPCD (+64%) in WT but not in ob/ob mice.

Concerning phosphatases, IPCD induced significant decreases in PTEN, MKP-3, and PP2C levels in WT mice (Fig. 7).

Interestingly, in ob/ob mice, PTEN, MKP-3, and PP2C were significantly increased with IPCD compared with their respective controls. Under control conditions (i.e., in the absence of postconditioning), the three phosphatase levels were significantly lower in ob/ob compared with WT mice.

DISCUSSION

The present study demonstrates for the first time that postconditioning fails to limit infarct size in obese mice. This lack of efficacy was accompanied by an impaired activation of the PI3K-Akt and ERK1/2 signaling pathways. To date, there is a consensus that the postconditioning stimulus must be applied immediately within the first minutes after the onset of reperfusion (25, 40). Concerning the algorithm, the number and the duration of cycles are of major importance (39). The postconditioning protocol used in the present study was determined on the basis of preliminary experiments and literature. We chose very short durations because their beneficial effects have been demonstrated in rats, i.e., periods of 10 s were more potent than those of 30 s (35). Concerning the number of cycles, a study performed in isolated mouse hearts reported that six postcon-
Conditioning cycles were more effective in improving posts ischemic systolic and diastolic functions than three postconditioning cycles (24). Accordingly, we investigated the cardioprotective effects of six cycles of 10-s reperfusion and 10-s occlusion. This postconditioning protocol conferred a rather strong cardioprotection in WT mice, because infarct size was significantly reduced by 58%. It is therefore unlikely that the failure of postconditioning to protect ob/ob mice against myocardial infarction is related to the algorithm. Similar alterations of cardioprotection with postconditioning have been reported in hypercholesterolemic rabbits and during aging (3, 19). Paradoxically, in our study, postconditioning tended to exacerbate the consequences of ischemia-reperfusion, because the postconditioned ob/ob mice elicited a greater mortality when the area at risk was similar to that in the other groups of mice. Therefore, we had to intentionally reduce this parameter in ob/ob mice subjected to postconditioning.

It is well known that in normal animals the activation of the so-called RISK pathway is essential for cardioprotection by postconditioning. It includes the PI3K-Akt and ERK1/2 cascades as well as their common downstream kinase target p70S6K1 (7, 38). In the present study, postconditioning significantly increased the phosphorylated state of Akt and ERK1/2 in WT mice. Consequently, we found that phosphorylation of p70S6K1 was increased at Thr389 (the site phosphorylated by Akt) and Thr421/Ser424 (the sites phosphorylated by ERK1/2). In contrast, postconditioning failed to increase the phosphorylation of Akt, ERK1/2, or p70S6K1 in ob/ob mice, confirming the infarct size results. We also investigated AMPK, a serine/threonine protein kinase that acts as a fuel sensor responsible for mediating the cellular adaptation to environmental stress (14). The phosphorylation of its threonine 172 site is essential for AMPK activation (34), which has been demonstrated to limit ischemic injury during posts ischemic reperfusion (30). Our results demonstrated that postconditioning activated AMPK by increasing its phosphorylation state in WT mice. Once again, in ob/ob mice postconditioning failed to elicit a similar effect.

The mechanisms responsible for this failure of postconditioning to activate the RISK pathway and to reduce infarct size in ob/ob mice remain unclear. Nevertheless, some hypotheses can be raised. At baseline (i.e., in the absence of ischemia-reperfusion) and under control conditions (i.e., without postconditioning), phosphorylation of most of the investigated kinases was significantly greater in ob/ob compared with WT mice. This might be the consequence of left ventricular hypertrophy, which has been reported to activate these signaling pathways (10). In addition, such increased phosphorylation of Akt has been reported previously in hepatic and muscular tissues of obese rats (23). This was accompanied by a concomitant and significant decrease in phosphatase levels such as PTEN, which dephosphorylates phosphatidylinositol 1,4,5-trisphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2) (26), MKP-3, a member of the dual-specificity phosphatases that plays a major role in ERK1/2 dephosphorylation (5), and PP2C, which is known to be a major enzyme that...
inactivates AMPK (9, 27). Nevertheless, this phenomenon should theoretically end up with a reduced infarct size in ob/ob compared with WT mice in the absence of postconditioning. However, our experiments revealed the opposite, because infarct size was significantly increased by 61% in ob/ob compared with WT mice, in agreement with previous studies performed in diabetic mice and obese rats (11, 20, 22). During postconditioning, we did not observe any increase in phosphorylation of Akt, ERK1/2, p70S6K1, or AMPK in ob/ob mice. Our results show for the first time that PTEN, MKP-3, and PP2C are significantly increased with postconditioning in ob/ob mice, while these phosphatases are significantly decreased in WT mice in the same situation. Interestingly, such a reduction in PTEN protein was previously reported with preconditioning in normal mice (4). Although we did not directly measure phosphatase activities, one could speculate that an enhanced phosphatase activity would be responsible for the lack of enhanced phosphorylation of protecting kinases and therefore contributes to the lack of beneficial effect of postconditioning. Interestingly, alterations in phosphatase activity or expression limit the efficacy of both preconditioning and postconditioning during aging (12, 29). Finally, we could also suggest that protein kinases and phosphatase determinants for infarct tolerance and transmission of cytoprotective signals may be different between C57BL/6J and ob/ob mice.

It should be stressed that our results could be explained by the lack of leptin, which exhibits cardioprotective properties by acting on the RISK pathway (32). Indeed, increase in infarct size under control conditions (i.e., in the absence of postconditioning) could result from the loss of protection afforded by leptin in ob/ob mice. In addition, although ob/ob mice exhibit the characteristics of obesity, it should be acknowledged that leptin mutation does not represent all causes of obesity in humans. Nevertheless, one would expect a decrease in Akt or ERK1/2 phosphorylation in ob/ob mice. In fact, we observed the opposite, because these phosphorylations were increased along with a greater infarct size in ob/ob compared with WT mice. Interestingly, administration of exogenous leptin in ob/ob mice has been demonstrated to worsen infarct volume after ischemia-reperfusion in the brain, and this effect was associated with an increased inflammatory response (36). Furthermore, postconditioning has been shown to be abrogated by hypercholesterolemia (cholesterol-enriched diet rabbits) (19), i.e., a model independent of leptin signaling disruption. Thus, although we cannot rule out that our results could be the consequence of the lack of leptin, we believe that the abrogation of postconditioning in ob/ob mice is instead due to obesity. In conclusion, the present study demonstrates that obesity impairs the ability of postconditioning to protect the heart against myocardial infarction. Further studies are necessary to determine how to restore the cardioprotective effect of postconditioning in this context of obesity.

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GRANTS

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REFERENCES


39. Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning’s protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation, *Basic Res Cardiol* 100: 57–63, 2005.

