

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

by

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*Running head:* Impaired ischemic postconditioning in obese mice

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## ABSTRACT

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 8-10 weeks old leptin-deficient obese mice (ob/ob) and compared to wild-type C57BL/6J mice (WT). 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 sacrificed at 10 min of reperfusion for Western blotting. In WT mice, IPCD reduced infarct size by 58% ( $33\pm 1\%$  vs  $14\pm 3\%$  for control and IDPC, respectively,  $p<0.05$ ) but failed to induce cardioprotection in ob/ob mice ( $53\pm 4\%$  vs  $56\pm 5\%$  for control and IPCD, respectively). In WT mice, IPCD significantly increased the phosphorylation of Akt (+77%), ERK 1/2 (+41%) and their common target p70S6K1 (+153% at Thr 389 and +57% at Thr 421/Ser 424). In addition, the phosphorylated AMPK to total AMPK ratio was also increased by IPCD in WT mice (+64%,  $p<0.05$ ). This was accompanied by decreases in PTEN, MKP-3 and PP2C levels. In contrast, IPCD failed to increase the phosphorylation state of all these kinases in ob/ob mice and the level of the 3 phosphatases were significantly increased. Thus, although IPCD reduces myocardial infarct size in healthy animals, its cardioprotective effect vanishes with obesity. The lack of enhanced phosphorylation by IPCD of Akt, ERK 1/2, p70S6K1 and AMPK might partly explain the loss of cardioprotection in this experimental model of obese mice.

*Key words:* Infarction, obesity, postconditioning, kinase, phosphatase.

## INTRODUCTION

Ischemic postconditioning has been described for the first time in dogs by Zhao et al. (42). These authors demonstrated that a series of repetitive cycles of brief reperfusion and reocclusion of the coronary artery applied at the onset of reperfusion after a prolonged ischemic event reduced infarct size. This finding has been confirmed in numerous investigations performed in mice (17, 21), rats (25, 35, 38), rabbits (2, 6, 7, 40), pigs (18) as well as in humans (8, 31, 33). To date, different mechanisms have been associated with this cardioprotective strategy (13, 41). Among them, a pro-survival signaling pathway so-called the reperfusion injury salvage kinase (RISK) pathway has been demonstrated to play a key role in postconditioning (7, 38). This pathway includes several pro-survival kinases such as phosphatidylinositol-3-OH-kinase (PI<sub>3</sub>K)-Akt, p42/44 extra-cellular signal-regulated kinases (ERK 1/2) or p70S6K1 (15).

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 co-morbidity.

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 so-

called ob/ob mice, characterized by hyperphagia, decreased energy expenditure and early onset of obesity (1).

## METHODS

### *Animals*

Male 8-10 weeks 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 h light-dark cycle and received standard rodent chow and drinking water *ad libitum*. The experiments were performed in accordance with the official regulations of the French Ministry of Agriculture.

### *Plasma measurements*

Plasma glucose, triglycerides, cholesterol and non-esterified fatty acids (NEFA) levels were measured using 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. The body temperature was maintained at 37°C. A left thoracotomy was performed in order to realize the 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 visualisation of hyperemic response and the chest was closed in layers.

The experimental protocol is shown in Figure 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 6 cycles of 10 s CAR/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 sacrificed 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 Evan's blue perfusion. The area at risk was identified using Evan's blue staining and the infarct area was identified by 2,3,5-triphenyltetrazolium chloride (TTC) staining. The area at risk was identified as the non-blue 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 area at risk.

#### *Tissue preparation*

Fresh ventricular tissues were placed in medium containing 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EGTA, 1 mM PMSF, 5 $\mu$ l/ml of protease inhibitor cocktail, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM sodium Na<sub>2</sub> $\beta$ -glycerol phosphate (Sigma-Aldrich, St. Louis, MO, USA), pH 7.4 at 4°C. The tissues were scissor minced and homogenized on ice using a Teflon Potter homogenizer. The homogenate was centrifuged at 17600 g for 30 min at 4°C to collect the cytosol. The cytosolic protein concentration was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

### *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-ERK 1/2 (Threonine 202/Tyrosine 204), phospho-p70S6K1 (Threonine 398), phospho-p70S6K1 (Threonine 421/Serine 424), phospho-AMPK (Threonine 172), total Akt, total ERK1/2, total p70S6K1, total AMPK, PTEN (all from Cell Signaling Technology, Danvers, MA, USA), MKP-3 (Epitomics, Burlingame, CA, USA) and 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, USA). Blots were finally incubated using the ECL detection system (ECL Western Blotting Substrate, Pierce, IL, USA) and exposed to ECL chemiluminescence film (Hyperfilm ECL, Amersham, Buckinghamshire, UK). Bands of interest were scanned and quantified in a blinded manner using gel analysis software ImageJ-1.37 (National Institute of Health, Bethesda, ML, USA).

### *Statistical analysis*

All values are expressed as mean  $\pm$  SEM. Comparisons were performed using Kruskal-Wallis analysis followed if necessary 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 as compared to 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 as compared to 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%).

### *Myocardial infarct sizes*

The areas at risk were similar among all groups excepted for the ob/ob mice subjected to postconditioning ( $44\pm 3\%$ , n=9;  $40\pm 3\%$ , n=9 and  $41\pm 4\%$ , n=8 for Control-WT, IPCD-WT and Control-ob/ob as compared to  $27\pm 2\%$ , n=7 for IPCD-ob/ob, respectively). 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 Figure 2, postconditioning significantly reduced infarct size by 58% in WT mice ( $33\pm 1\%$  vs  $14\pm 3\%$  for Control-WT and IPCD-WT, respectively). In contrast, this strategy failed to protect ob/ob mice against myocardial infarction ( $53\pm 4\%$  vs  $56\pm 5\%$  for Control-ob/ob and IPCD-ob/ob, respectively). Interestingly, in control conditions, infarct size was significantly increased by 61% in ob/ob as compared to WT mice.



### *Baseline analysis*

As illustrated in Figure 3, phospho-Akt (Ser 473)/Akt, phospho-ERK 1/2(Thr 202/Tyr 204)/ERK 1/2 and phospho-AMPK (Thr 172)/AMPK ratios were significantly increased in ob/ob (n=5) as compared to 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 as compared to WT mice.

### *Effect of IPCD on PTEN, MKP-3, PP2C and the phosphorylation of Akt, ERK 1/2, p70S6K1 and AMPK*

The effects of IPCD on the phosphorylation states of pro-survival kinases Akt, ERK 1/2 and p70S6K1 as well as AMPK were investigated in Control-WT (n=3), IDPC-WT (n=3), Control-ob/ob (n=4) and IDPC-ob/ob (n=4) mice.

As illustrated in Figure 4A, IPCD induced a significant increase in the phospho-Akt (Ser 473)/Akt ratio (+ 77%) in WT mice. Similarly, in WT mice, IPCD significantly increased the phosphorylated ERK 1/2 (Thr 202/Tyr 204)/ERK 1/2 ratio (+ 41%) (Figure 4B). In contrast, these effects were not observed in ob/ob mice. In control conditions (*i.e.*, in the absence of IPCD), these ratios were significantly greater in ob/ob as compared to WT mice.

As p70S6K1 is known to be phosphorylated at Threonine 389 by activated Akt as well as at both Threonine 421 and Serine 424 by activated ERK 1/2, we examined these phosphorylations. As illustrated in Figure 5, IPCD induced significant increases in phosphorylated p70S6K1 (Thr 389)/p70S6K1 (+153%) and phosphorylated p70S6K1 (Thr 421/Ser 424)/p70S6K1 ratios (+57%) in WT mice. This strategy failed to elicit similar effects in ob/ob mice.

As illustrated in Figure 6, the phosphorylated AMPK (Thr 172)/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. Interestingly in ob/ob mice, PTEN, MKP-3 and PP2C were significantly increased with IPCD as compared to their respective control. In control conditions (*i.e.*, in the absence of postconditioning), the three phosphatase levels were significantly lower in ob/ob as compared to 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 PI<sub>3</sub>K-Akt and ERK 1/2 signaling pathways. To date, there is a consensus indicating 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 rather chose very short durations as 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 mice hearts reported that 6 postconditioning cycles were more effective in improving post-ischemic systolic and diastolic functions than 3 postconditioning cycles (24). Accordingly, we investigated the cardioprotective effects of 6 cycles of 10 s reperfusion / 10 s occlusion. This postconditioning protocol conferred a rather strong cardioprotection in WT mice as 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 as the postconditioned ob/ob mice elicited a greater mortality when the area at risk was similar to the other groups of mice. So, we had to intentionally reduce this parameter in ob/ob mice subjected to postconditioning.

It is well known in normal animals that the activation of the so-called RISK pathway is essential for cardioprotection by postconditioning. It includes the PI<sub>3</sub>K-Akt and ERK 1/2

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 ERK 1/2 in WT mice. Consequently, we found that phosphorylation of p70S6K1 was increased at Thr 389 (the site phosphorylated by Akt) and Thr 421/Ser 424 (the sites phosphorylated by ERK 1/2). In contrast, postconditioning failed to increase the phosphorylation of Akt, ERK 1/2 or p70S6K1 in ob/ob mice, comforting the infarct size results. We also investigated AMPK, a serine/threonine protein kinase, which 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 postischemic 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 hypothesis can be raised. At baseline (*i.e.*, in the absence of ischemia-reperfusion) and in control conditions (*i.e.*, without postconditioning), phosphorylation of most of the investigated kinases was significantly greater in ob/ob as compared to 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 previously reported 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 PIP<sub>3</sub> to PIP<sub>2</sub> (26), MKP-3, a member of the dual-specificity phosphatases which plays a major role in ERK 1/2 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 as compared to WT mice in the

absence of postconditioning. However our experiments revealed the opposite as infarct size was significantly increased by 61% in ob/ob as compared to 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, ERK 1/2, 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 reduction in PTEN protein has been 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 has contributed 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 activating the RISK pathway (32). Indeed, increase in infarct size in 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 ERK phosphorylation in ob/ob mice. In fact, we observed the opposite as these phosphorylations were increased along with a greater infarct size in ob/ob as compared to WT mice. Interestingly, administration of exogenous leptin in ob/ob mice has been

demonstrated to worsen infarct volume following ischemia-reperfusion in the brain and this effect was associated with an increased inflammatory response (36). Furthermore, postconditioning has been shown to be abrogated with 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 rather 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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## LEGEND OF FIGURES

### *Figure 1*

Experimental protocols used for investigating the effects of ischemic postconditioning (IPCD) in wild-type and ob/ob mice (CAO, coronary artery occlusion; CAR, coronary artery reperfusion).

### *Figure 2*

Infarct size (expressed as percentage of the area at risk) measured in wild-type (WT) and ob/ob mice subjected to 30 min of coronary artery occlusion and 24 h of reperfusion in the absence or presence of postconditioning (Control and IPCD, respectively). Open circles represent individual values and closed circles indicate the average. \* $p < 0.05$  vs Control; †  $p < 0.05$  vs respective WT.

### *Figure 3*

Western blot analysis of Akt and its phosphorylated form at Ser 473 (panel A), ERK 1/2 and its phosphorylated forms at Thr 202 and Tyr 204 (panel B), p70S6K1 and its phosphorylated form at Thr 389 due to the effect of Akt (panel C), p70S6K1 and its phosphorylated form at Thr 421 and Ser 424 due to the effects of ERK1/2 (panel D), AMPK and its phosphorylated form (panel E), PTEN (panel F), MKP-3 (panel G) and PP2C (panel H) in wild-type (WT) and ob/ob mice performed at baseline (*i.e.*, in the absence of ischemia-reperfusion). Values are expressed as mean  $\pm$  SEM (n=5 per group for WT mice and n=5 per group for ob/ob mice). \* $p < 0.05$  vs WT.

*Figure 4*

Western blot analysis of Akt and its phosphorylated form at Ser 473 (panel A) as well as ERK 1/2 and its phosphorylated forms at Thr 202 and Tyr 204 (panel B) in wild-type (WT) and ob/ob mice submitted to 30 min of coronary artery occlusion and 10 min of reperfusion in the absence (Control) or presence of postconditioning (IPCD). Values are expressed as mean  $\pm$  SEM (n=3 per group for WT mice and n=4 per group for ob/ob mice). \* $p$ <0.05 vs respective Control; †  $p$ <0.05 vs respective WT.

*Figure 5*

Western blot analysis of p70S6K1 and its phosphorylated forms. Panel A: Thr 389 due to the effect of Akt; panel B: Thr 421 and Ser 424 due to the effects of ERK 1/2 in wild-type (WT) and ob/ob mice submitted to 30 min of coronary artery occlusion and 10 min of reperfusion in the absence (Control) or presence of postconditioning (IPCD). Values are expressed as mean  $\pm$  SEM (n=3 per group for WT mice and n=4 per group for ob/ob mice). \* $p$ <0.05 vs respective Control; †  $p$ <0.05 vs respective WT.

*Figure 6*

Western blot analysis of AMPK and its phosphorylated form (Thr 172) in wild-type (WT) and ob/ob mice submitted to 30 min of coronary artery occlusion and 10 min of reperfusion in the absence (Control) or presence of postconditioning (IPCD). Values are expressed as mean  $\pm$  SEM (n=3 per group for WT mice and n=4 per group for ob/ob mice). \* $p$ <0.05 vs respective Control; †  $p$ <0.05 vs respective WT.



*Figure 7*

Western blot analysis of PTEN (panel A), MKP-3 (panel B) and PP2C (panel C) in wild-type (WT) and ob/ob mice submitted to 30 min of coronary artery occlusion and 10 min of reperfusion in the absence (Control) or presence of postconditioning (IPCD). Values are expressed as mean  $\pm$  SEM (n=3 per group for WT mice and n=4 per group for ob/ob mice).

\* $p < 0.05$  vs respective Control; †  $p < 0.05$  vs respective WT.

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

|                      | <b>WT (n=6)</b> | <b>ob/ob (n=6)</b> |
|----------------------|-----------------|--------------------|
| Glycemia (mM)        | 12.17 ± 0.63    | 24.69 ± 2.46 *     |
| Triglyceridemia (mM) | 0.89 ± 0.07     | 0.68 ± 0.07        |
| Cholesterolemia (mM) | 2.78 ± 0.12     | 4.81 ± 0.17 *      |
| NEFA (mM)            | 1.28 ± 0.12     | 1.18 ± 0.08        |

Values are expressed as mean ± SEM. \* $p < 0.05$  vs WT.

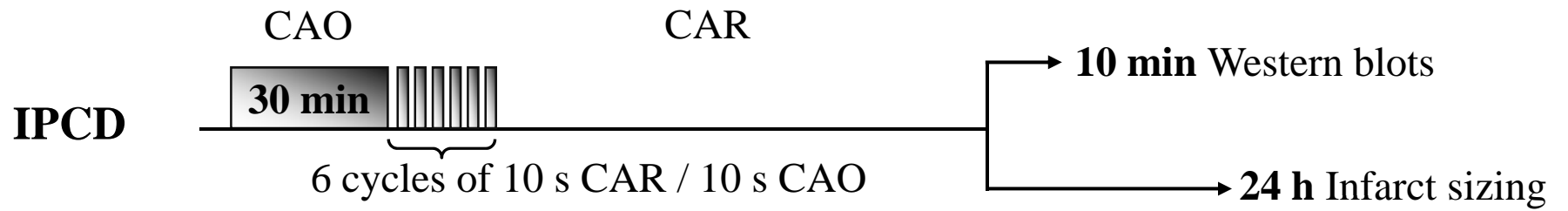
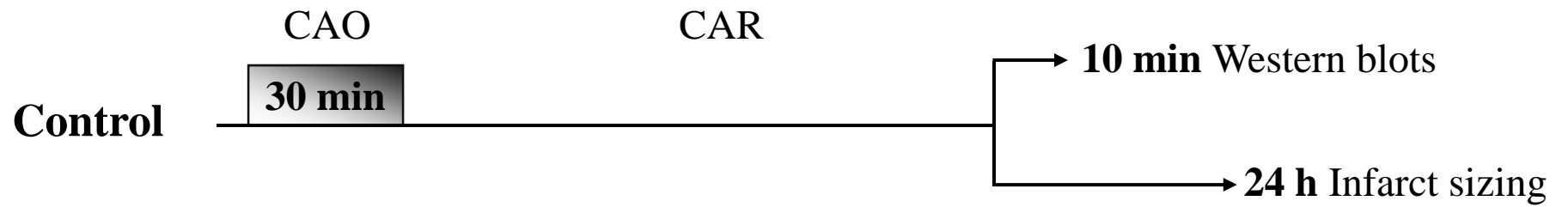
NEFA: non-esterified fatty acids.

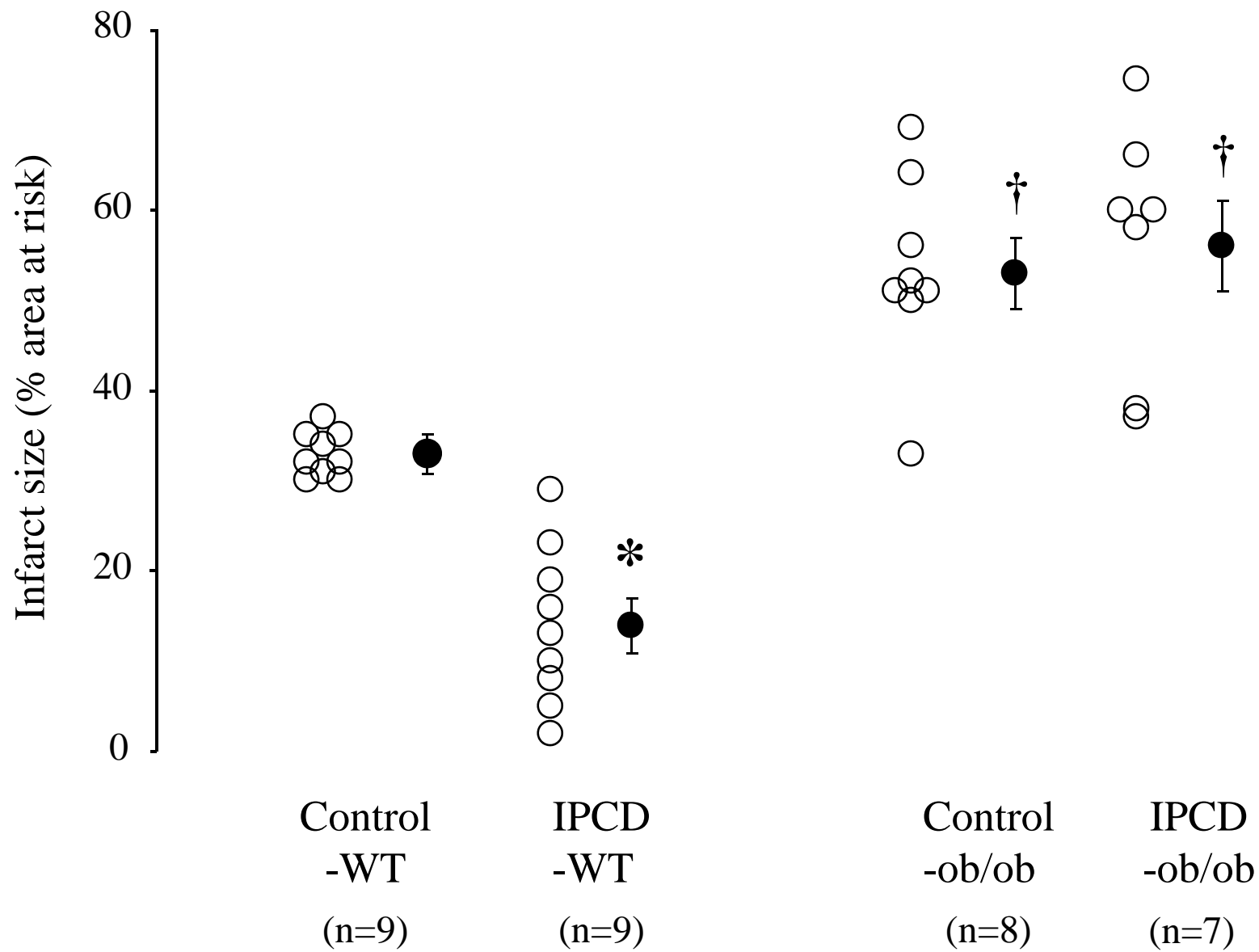
**Table 2: General parameters in WT and ob/ob mice.**

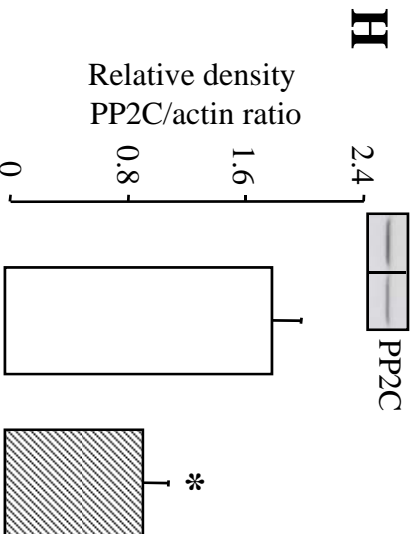
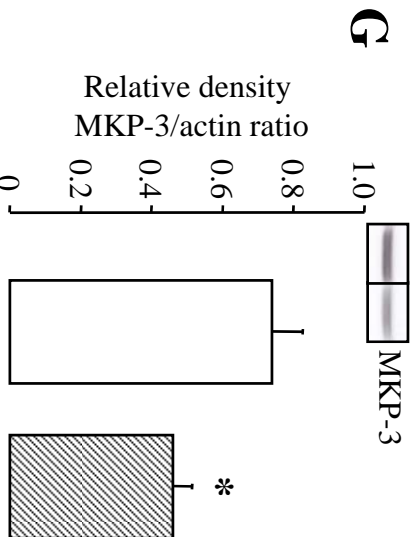
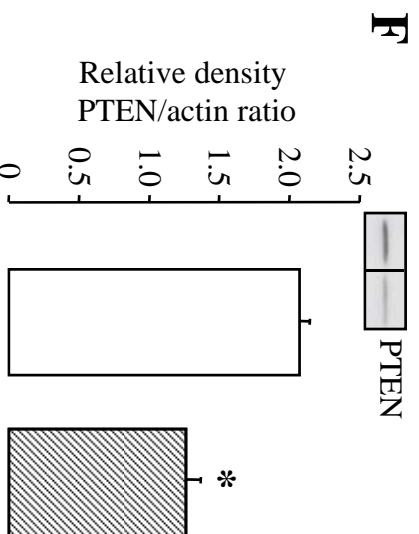
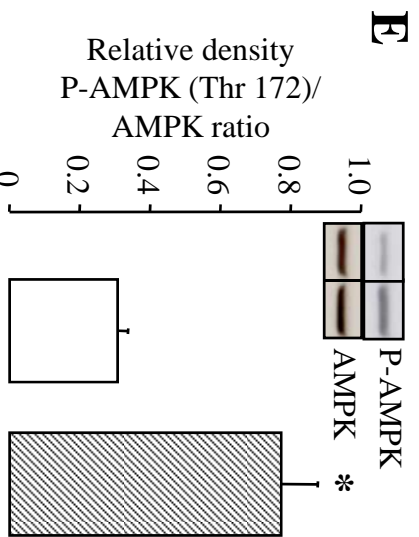
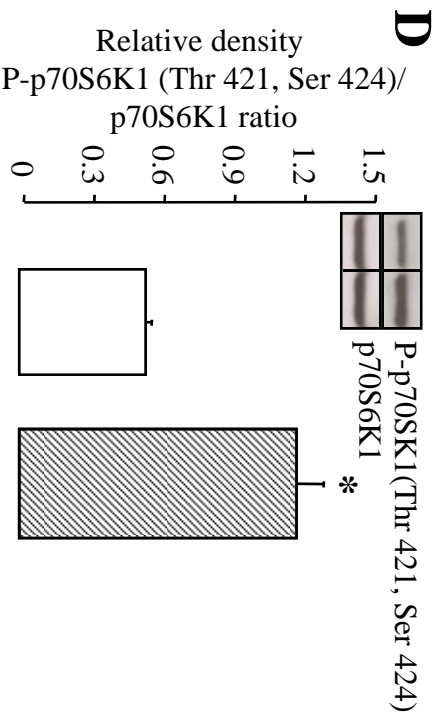
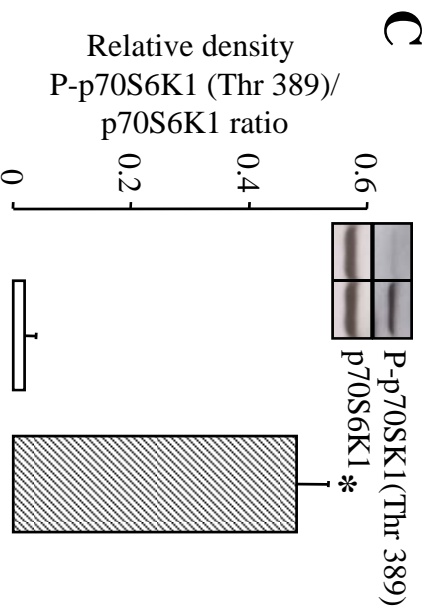
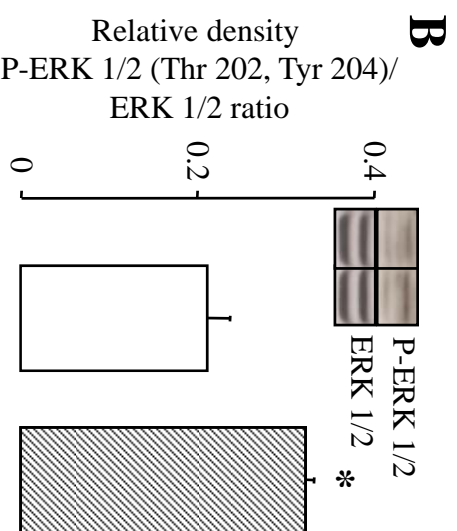
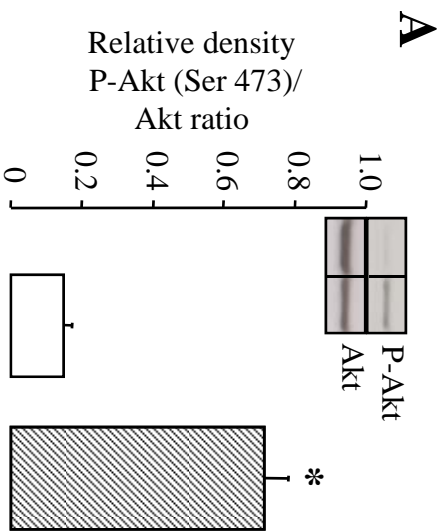
|   | WT            |            | ob/ob         |              |
|---|---------------|------------|---------------|--------------|
|   | Control (n=9) | IPCD (n=9) | Control (n=8) | IPCD (n=7)   |
| Body weight (g)                               | 25.5 ± 0.7    | 23.4 ± 0.3 | 48.2 ± 1.2 †  | 46.0 ± 1.9 † |
| Left ventricular weight (mg)                  | 80.8 ± 2.1    | 75.2 ± 2.4 | 88.2 ± 2.6 †  | 87.5 ± 2.5 † |
| Tibial length (mm)                            | 17.6 ± 0.1    | 17.1 ± 0.2 | 17.3 ± 0.2    | 17.3 ± 0.3   |
| Left ventricular weight/tibial length (mg/mm) | 4.6 ± 0.1     | 4.4 ± 0.2  | 5.1 ± 0.2 †   | 5.1 ± 0.2 †  |

Values are expressed as mean ± SEM. †  $p < 0.05$  vs respective WT.

IPCD: ischemic postconditioning.







□ WT

▨ ob/ob

