

Supplementary Materials for

**Failure of isoflurane cardiac preconditioning in obese type 2  
diabetic mice involves aberrant regulation of microRNA-21,  
endothelial nitric oxide synthase, and mitochondrial complex I**

Zhi-Dong Ge,\* Yingchuan Li, Shigang Qiao, Xiaowen Bai,  
David C. Warltier, Judy R. Kersten, Zeljko J. Bosnjak, Mingyu Liang

\*Corresponding author. E-mail: [Wilson.ge99@gmail.com](mailto:Wilson.ge99@gmail.com) (Z.-D.G.)

The Supplementary Materials include:

Materials and Methods: myocardial ischemia/reperfusion injury *in vivo*.

Materials and Methods: determination of group size of *in vivo* myocardial  
ischemia/reperfusion injury.

Results: Figure S1 Db/db mice developed obesity and hyperglycemia.

Results: Isoflurane preconditioning decreased infarct size in C57BL/6 mice  
subjected to myocardial ischemia/reperfusion injury *in vivo* (Figure S2)

Results: Isoflurane preconditioning did not decrease infarct size in db/db mice  
subjected to ischemia/reperfusion injury *in vivo* (Figure S3)

Results: Effects of isoflurane on heart rate and coronary flow rate during  
reperfusion in Langendorff-perfused hearts (Figure S4)

Results: Western blot band of dimeric endothelial nitric oxide synthase (Figure  
S5)

Results: Survival and parametric data of *in vivo* myocardial ischemia/reperfusion injury

## **Materials and Methods**

### ***Myocardial ischemia/reperfusion injury in vivo***

*Surgical Preparation.* C57BL/6 and db/db mice at 12-14 weeks of age were anesthetized by intraperitoneal injection of sodium pentobarbital (80 mg/kg) and ventilated with room air supplemented with 100% oxygen at a rate of ~102 breaths per minute with a tidal volume of ~225  $\mu$ l using a rodent ventilator (Hugo Sachs Elektronik, Harvard Apparatus, Germany). Under a dissecting microscope (Thermo Fisher Scientific Inc., Pittsburgh, PA), the left common carotid artery was cannulated with a polyethylene-10 tubing for sampling arterial blood. A 25  $\mu$ l of arterial blood was taken through the catheter after a stabilization period of 20 min (baseline) and 30 min after reperfusion. Arterial blood gas analysis was performed using a blood gas analyzer (ABL-725 Radiometer). Arterial blood gas tensions and acid-base status were maintained within a physiological range (pH between 7.35 and 7.45, PaCO<sub>2</sub> between 35 and 45 mmHg, and PaO<sub>2</sub> between 120 and 180 mmHg) by adjusting the respiratory rate or tidal volume. An 8-0 nylon suture is passed below the left main descending coronary artery 1-3 mm from tip of the normally positioned left auricle. Myocardial ischemia is induced by tying the suture over a piece of rolled wetted gauze for 30 min, and reperfusion is initiated by loosening the suture.<sup>1</sup> Successful performance of coronary artery occlusion and reperfusion is verified by visual inspection (for example, by noting the development of a pale color in the distal myocardium upon occlusion and the return of a bright red color due to hyperemia after release) and by observing widening of QRS

wave and changes of ST-segment (depressed ST-segment during ischemia and elevated ST-segment after reperfusion) on the electrocardiogram. Body temperature was maintained between 36.8 °C and 37.5 °C throughout the experiment by using a heating pad (Model TC-1000). The infarct area was delineated by perfusing the coronary arteries with 2,3,5-triphenyltetrazolium chloride via the aortic root, and the area at risk was delineated by perfusing phthalo blue dye (Heucotech Ltd., Fairless Hill, PA) into the aortic root after tying the coronary artery at the site of previous occlusion. As a result of these procedures, the nonischemic portion of the LV was stained black or dark blue. Viable myocardium within the area at risk was stained bright red, and infarcted tissue was white.

*Experimental protocols.* The following two protocols were used to test the effect of isoflurane on myocardial infarct size in C57BL/6 and db/db mice. Protocol 1 determined the concentration-dependent effects of isoflurane on infarct size in C57BL/6 mice randomly assigned to 4 experimental groups (10 mice/group): control, isoflurane<sub>0.8</sub>, isoflurane<sub>1.4</sub>, and isoflurane<sub>2.0</sub>. After instrumentation was completed, all mice were stabilized for 30 minutes and subjected to 30 minutes of coronary artery occlusion followed by 2 h of reperfusion. Isoflurane at the concentrations of 0.8%, 1.4%, or 2.0% was administered for 30 minutes via an isoflurane-specific vaporizer (Ohio Medical Instruments, Madison, WI) followed by a 15 minutes period of washout prior to coronary artery occlusion. The concentrations of isoflurane in mouse serum 20 minutes after administration of isoflurane were determined by a gas chromatography (1.4% isoflurane was equivalent to 1.0 minimum alveolar concentration). Control mice received no isoflurane. Heart rate was monitored from the electrocardiogram. Protocol 2

determined the effect of 1.4% isoflurane on infarct size in C57BL/6 and db/db mice subjected to 30 minutes of myocardial ischemia followed by 2 h of reperfusion. The mice were divided into the following 4 groups (n=10 C57BL/6 or 12 db/db mice/group): control, db/db, isoflurane<sub>1.4</sub>, and db/db+isoflurane<sub>1.4</sub>. All mice were stabilized for 30 minutes and subjected to 30 minutes of coronary artery occlusion followed by 2 h of reperfusion. Isoflurane at a concentration of 1.4% was given for 30 minutes prior to coronary artery occlusion in C57BL/6 (isoflurane<sub>1.4</sub> group) or db/db mice (db/db+isoflurane<sub>1.4</sub>).

#### ***Determination of group size of in vivo myocardial ischemia/reperfusion injury***

In *in vivo* experiments of myocardial ischemia/reperfusion injury, group size was determined by using a power analysis of means from published results as well as our experience to estimate the number of animals needed to test the null hypothesis. Infarct size in C57BL/6 mice without the treatment of isoflurane is typically  $54\pm 10\%$ , and infarct size in C57BL/6 mice preconditioned with 1.4% of isoflurane is around  $36\pm 10\%$ .<sup>2</sup> Based upon an average standard deviation of 10% from our prior work with ischemia/reperfusion injury in C57BL/6 mice,<sup>3,4</sup> an  $n = 8$  per group will allow for detection of a difference between groups of up to 16% at  $P < 0.05$ . In our previous studies, 2 out of 10 C57BL/6 mice and 4 out of 10 db/db mice undergoing myocardial ischemia/reperfusion injury were usually unsuccessful due to death during ischemia or reperfusion or unsuccessful heart staining. Thus, 10 C57BL/6 and 12 db/db mice per group were needed for these experiments.

## Results

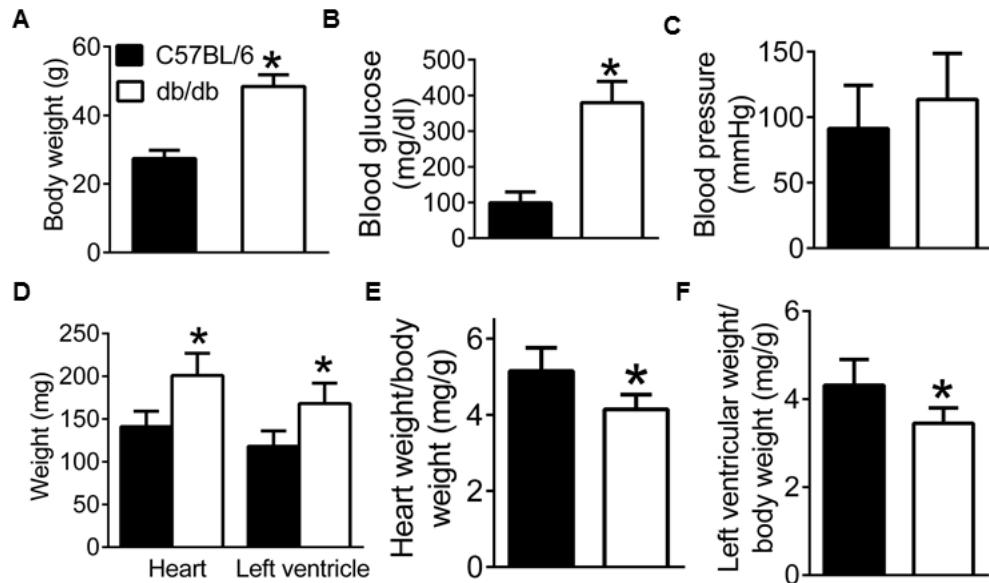


Figure S1 Db/db mice at 12-14 weeks of age developed obesity and hyperglycemia. A: body weight (mean  $\pm$  SD) (n=10 mice/group); B: fasting blood glucose (n=10 mice/group); C: mean arterial blood pressure (n=8 mice/group); D: heart weight and left ventricular weight (n=10 mice/group); E: the ratio of heart weight/body weight (n=10 mice/group); F: the ratio of left ventricular weight/body weight (n=10 mice/group). \*P<0.05 versus C57BL/6 mice.

### ***Isoflurane decreased infarct size in C57BL/6 mice subjected to myocardial ischemia/reperfusion injury in vivo***

Area at risk was  $51 \pm 11\%$  (n=10 mice) of the left ventricle, and infarct size was  $56 \pm 10\%$  of area at risk in C57BL/6 mice subjected to 30 min of coronary artery occlusion followed by 2 h of reperfusion (Figure S2). Isoflurane at a concentration of 0.8% administered prior to coronary artery occlusion did not significantly change area at risk

and infarct size ( $P > 0.05$  between Isoflurane<sub>0.8</sub> and control groups). Interestingly, increased concentrations of isoflurane to 1.4% and 2.0% did not significantly impacted area at risk but decreased infarct size to  $38 \pm 10\%$  and  $36 \pm 10\%$  ( $P < 0.05$ ,  $n = 8$  mice/group), respectively.

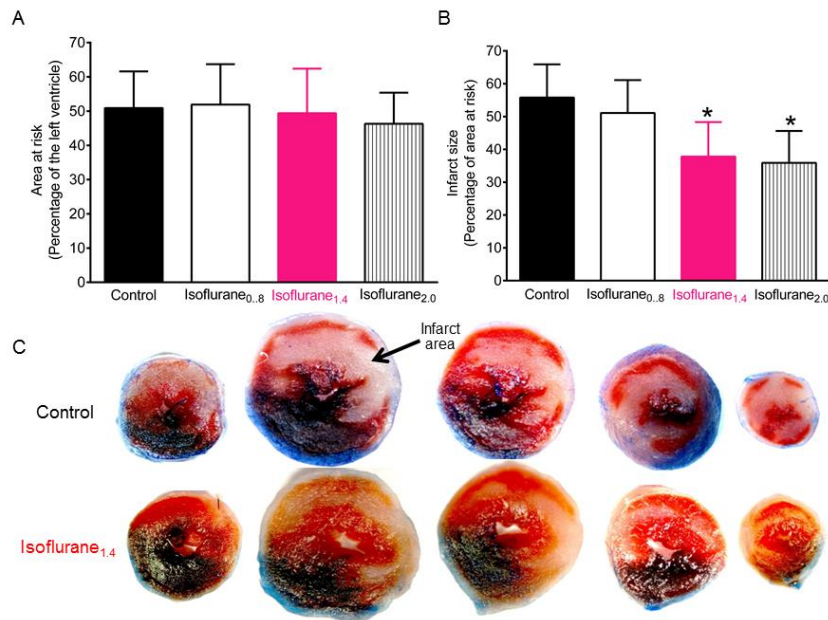


Figure S2 Isoflurane preconditioning reduced infarct size in C57BL/6 mice subjected to ischemia/reperfusion injury. A: area at risk expressed as a percentage of the left ventricle (mean  $\pm$  SD); B: infarct size expressed as a percentage of area at risk. C57BL/6 mice were given isoflurane at 0% (control,  $n = 10$  mice), 0.8% (Isoflurane<sub>0.8</sub>,  $n = 8$  mice), 1.4% (isoflurane<sub>1.4</sub>,  $n = 8$  mice), or 2.0% (isoflurane<sub>2.0</sub>,  $n = 8$  mice) and were subsequently subjected to 30 minutes of coronary artery occlusion and 2 h of perfusion; C: representative images of heart sections stained by 2,3,5-triphenyltetrazolium chloride and phthalol blue. Top: five slices from a C57BL/6 control mouse; bottom: five slices from a C57BL/6 mouse given 1.4% isoflurane prior to coronary artery occlusion and reperfusion *in vivo*. Non-area at risk was stained black. Within area at risk,

noninfarcted, viable tissue was stained red, whereas the infarcted region was left unstained. \*P<0.05 versus control groups.

***Isoflurane preconditioning did not decrease infarct size in db/db mice subjected to ischemia/reperfusion injury in vivo***

Figure S3 demonstrates the effect of isoflurane on myocardial infarct size in C57BL/6 and db/db mice. In C57BL/6 mice, 20 minutes of ischemia followed by 2 h of reperfusion resulted in an area at risk of  $50\pm 9\%$  with an infarct size of  $35\pm 9\%$  of area at risk (n=9 mice/group). Area at risk was comparable between db/db and C57BL/6 mice. However, infarct size was bigger in db/db ( $51\pm 9\%$ , n=8 mice) than C57BL/6 mice (P<0.05). In C57BL/6 mice, 30 minutes of ischemia followed by 2 h of reperfusion resulted in an area at risk of  $48\pm 12\%$  with an infarct size of  $53\pm 12\%$  of area at risk (n=10 mice/group). Infarct size was elevated to  $62\pm 11\%$  (n=8 mice) in db/db mice. ISO at a concentration of 1.4% significantly decreased infarct size to  $37\pm 10\%$  (n=9 mice) in 30 minutes of ischemia followed by 2 h of reperfusion in C57BL/6 mice. In contrary, there were no significant differences in infarct size between db/db+isoflurane<sub>1.4</sub> and control or db/db groups (P>0.05, n=8-10 mice/group).

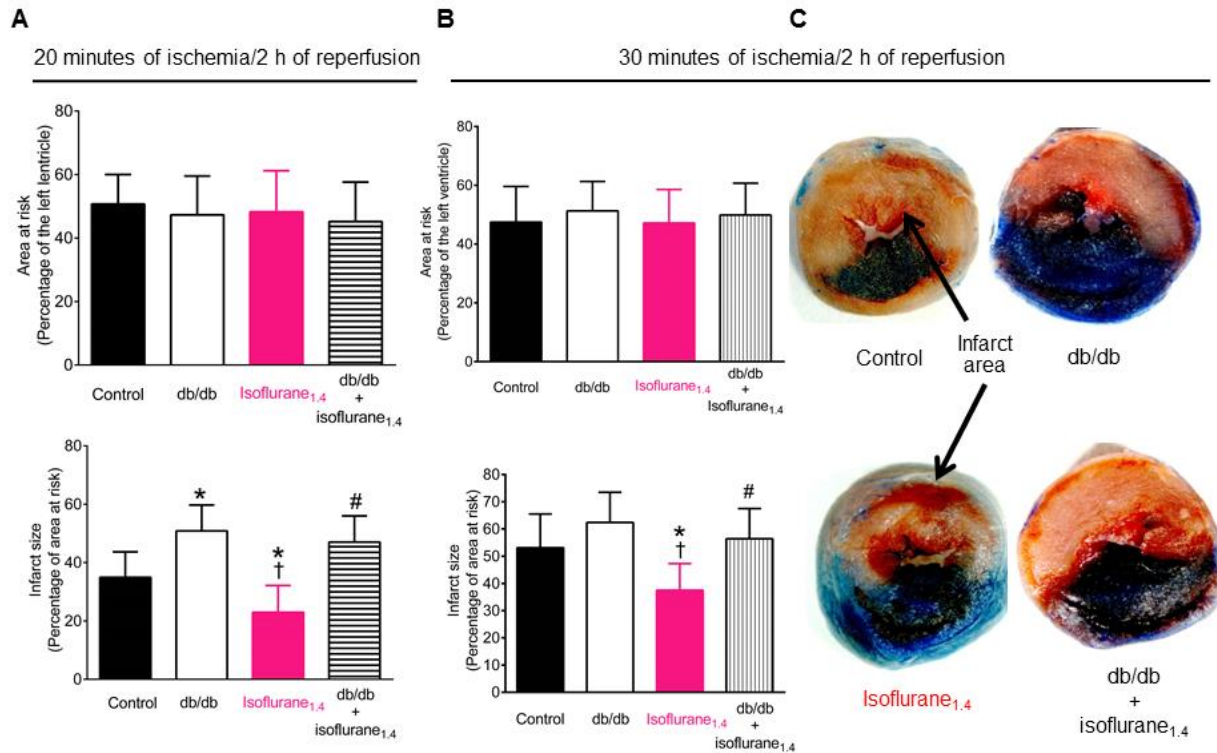


Figure S3 Isoflurane preconditioning decreased myocardial infarct size in C57BL/6 but not db/db mice subjected to ischemia/reperfusion injury. A: area at risk (top) and infarct size (bottom) in db/db and C57BL/6 mice subjected to 20 minutes of coronary artery occlusion followed by 2 h or reperfusion (mean  $\pm$  SD). Control (n=10 mice), C57BL/6 mice undergoing coronary artery occlusion for 20 minutes followed by 2 h of reperfusion; db/db (n=8 mice), db/db mice undergoing coronary artery occlusion for 20 minutes followed by 2 h of reperfusion; Isoflurane<sub>1,4</sub> (n=9 mice), C57BL/6 mice treated with 1.4% of isoflurane prior to 20 minutes of coronary artery occlusion followed by 2 h of reperfusion; db/db+isoflurane<sub>1,4</sub> (n=8 mice), db/db mice treated with 1.4% of isoflurane before 20 min of coronary artery occlusion followed by 2 h of reperfusion; B: area at risk (top) and infarct size (bottom) in db/db and C57BL/6 mice subjected to 30 minutes of coronary artery occlusion followed by 2 h or reperfusion; C: representative



photographs of heart sections stained with 2,3,5-triphenyltetrazolium chloride and phthalol blue dye. The hearts were stained with 2,3,5-triphenyltetrazolium chloride and phthalol blue dye to delineate area at risk and infarct size after completion of reperfusion after ischemia. Control (n=10 mice), C57BL/6 mice undergoing coronary artery occlusion for 30 minutes followed by 2 h of reperfusion; db/db (n=8 mice), db/db mice undergoing coronary artery occlusion for 30 minutes followed by 2 h of reperfusion; Isoflurane<sub>1.4</sub> (n=9 mice), C57BL/6 mice treated with 1.4% of isoflurane prior to 30 minutes of coronary artery occlusion followed by 2 h of reperfusion; db/db+isoflurane<sub>1.4</sub> (n=8 mice), db/db mice treated with 1.4% of soflurane before 30 minutes of coronary artery occlusion followed by 2 h of reperfusion. \*P<0.05 versus control groups, †P<0.05 versus db/db groups, #P<0.05 versus isoflurane<sub>1.4</sub> groups.

***Effects of isoflurane on heart rate and coronary flow rate during reperfusion in Langendorff-perfused hearts***

Figure S4 demonstrates heart rate and coronary flow rate of Langendorff-perfused mouse hearts subjected to global ischemia/reperfusion injury with or without the treatment of isoflurane. The values of heart rate and coronary flow rate at baseline were smaller in db/db than control groups (P<0.05). Global ischemia for 30 minutes resulted in the cessation of the contraction and relaxation of the hearts. With reperfusion, contraction and relaxation were gradually restored in all mouse hearts. Hear rate and coronary flow rate during reperfusion were not significantly changed by isoflurane in C57BL/6 mice (P>0.05 between isoflurane and control groups). Heart rate was slower in db/db than control groups from 10 minutes to 2 h after reperfusion (P<0.05), and coronary flow rate was smaller in db/db than control groups 10 and 30 min after reperfusion. There were significant decreases in heart rate and coronary flow rate

in db/db+isoflurane from 30 minutes to 2 h after reperfusion compared with isoflurane groups. There were not significant differences in heart rate and coronary flow rate between db/db+isoflurane<sub>1,4</sub> and db/db groups at baseline and during reperfusion ( $P>0.05$ ).

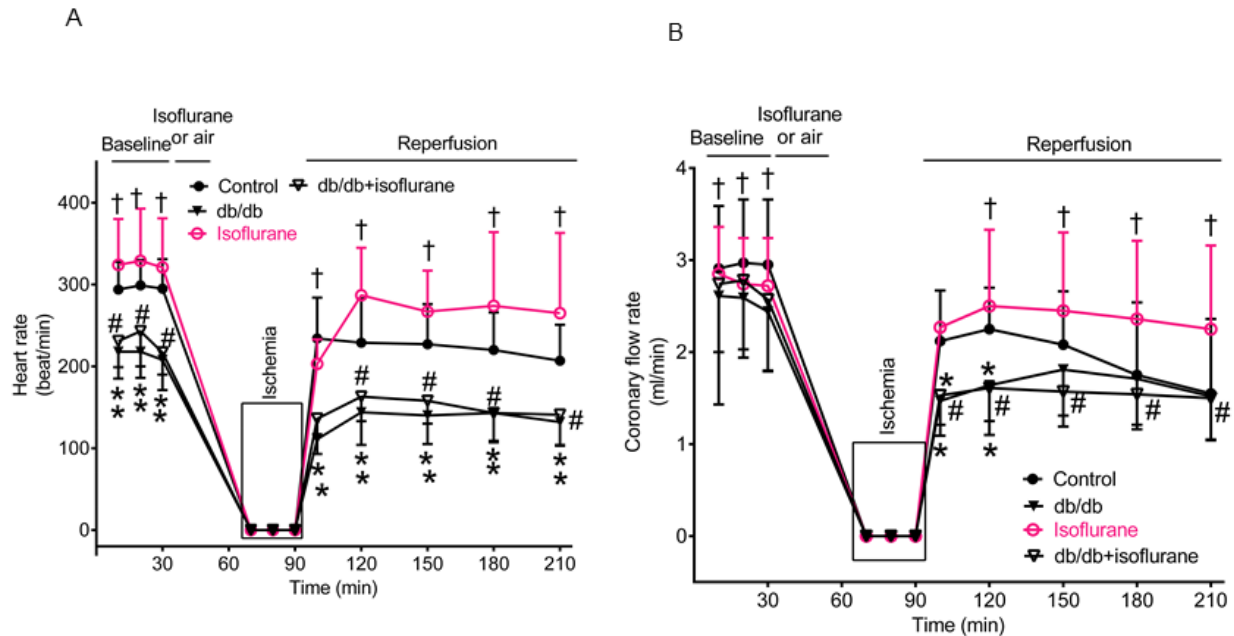


Figure S4 Changes in heart rate (A) and coronary flow rate (B) in db/db and C57BL/6 mice at baseline and during ischemia and reperfusion in langendorff-perfused hearts. Control, C57BL/6 mouse hearts undergoing ischemia/reperfusion injury; db/db, C57BL/6 mouse hearts undergoing ischemia/reperfusion injury; Isoflurane, C57BL/6 mouse hearts treated with 1.4% of isoflurane before ischemia; db/db+isoflurane, db/db mouse hearts treated with 1.4% of isoflurane before ischemia. \* $P<0.05$  versus control groups, † $P<0.05$  versus db/db groups, # $P<0.05$  versus isoflurane groups (n=8-10 mice/group).

### **Western blot band of dimeric endothelial nitric oxide synthase**

The molecular weight of monomeric endothelial nitric oxide synthase is 133 kDa. Theoretically, the molecular weight of dimeric endothelial nitric oxide synthase is 266 kDa. The anticipated band size of dimeric and monomeric endothelial nitric oxide

synthase on Western blot is 266 kDa and 133 kDa, respectively. However, the migration of proteins is affected by multiple factors, including post-translational modification, post-translational cleavage, splice variants, and relative charge, etc. In order to determine actual size of Western blot band of dimeric endothelial nitric oxide synthase, we analyzed the expression of endothelial nitric oxide synthase in mouse myocardium from the following groups: a sham-operated C57BL/6 mouse (sham WT), a sham-operated db/db mouse (sham db), a C57BL/6 mouse underlying myocardial ischemia/reperfusion injury (I/R WT), a db/db mouse underlying myocardial ischemia/reperfusion injury (I/R db), a C57BL/6 mouse receiving isoflurane preconditioning prior to myocardial ischemia/reperfusion injury (ISO WT), a db/db mouse receiving isoflurane preconditioning prior to myocardial ischemia/reperfusion injury (ISO db), and three fresh C57BL/6 mouse hearts. As demonstrated in Figure S5, these tissues had a band at 230 kDa, and this band disappeared after myocardial tissues were boiled. These results indicate that 230 kDa band is dimeric endothelial nitric oxide synthase. Our results are consistent with a previous study showing that actual Western blot band sizes of monomeric and dimeric endothelial nitric oxide synthase are 130 and 230 kDa, respectively, in obese mice.<sup>5</sup> It is likely that some factors from obesity and/or diabetes may modify endothelial nitric oxide synthase dimers or/and impact the migration of dimeric endothelial nitric oxide synthase.

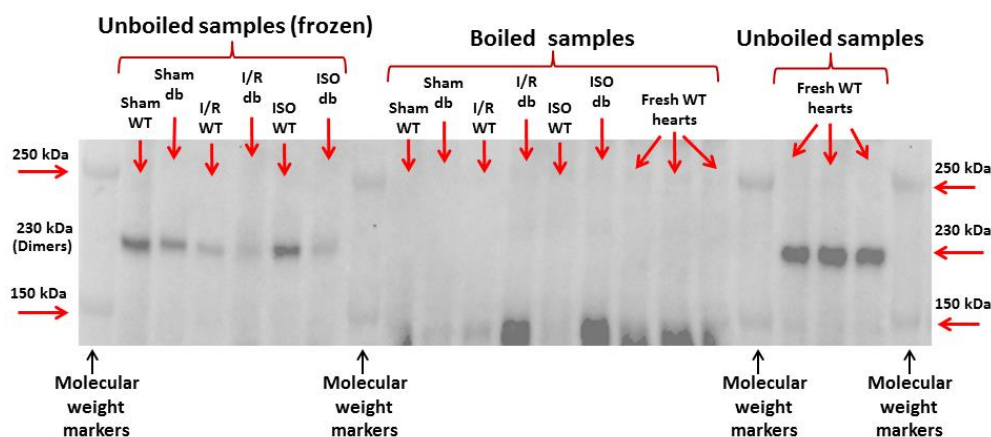


Figure S5 Representative Western blot bands showing the expression of dimeric endothelial nitric oxide synthase (230 kDa).

### ***Survival and parametric data of in vivo myocardial ischemia/reperfusion injury***

A total of 90 C57BL/6 and 70 db/db mice entered the protocol of 30 min of *in vivo* myocardial ischemia followed by 2 h of reperfusion. Ten C57BL/6 and 24 db/db mice died during the period of ischemia or reperfusion. Consequently, 80 C57BL/6 and 46 db/db mice successfully completed the *in vivo* ischemia/reperfusion experimental protocol. Among 20 C57BL/6 and 20 db/db mice that were used to determine area at risk and infarct size, coronary vascular system was leaky in 1 C57BL/6 and 4 db/db mice, when 2,3,5-triphenyltetrazolium chloride or phthalo blue dye was perfused into the myocardium. These 5 mice were excluded from the infarct assessment data. Seventy-nine C57BL/6 and 42 db/db mice successfully completed the *in vivo* ischemia/reperfusion experimental protocol were included for data analysis.

### **References**

1. Ge ZD, Peart JN, Kreckler LM, Wan TC, Jacobson MA, Gross GJ, Auchampach JA: CI-IB-MECA [2-chloro-N<sup>6</sup>-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide] reduces ischemia/reperfusion injury in mice by activating the A<sub>3</sub> adenosine receptor. *J Pharmacol Exp Ther* 2006; 319: 1200-10
2. Qiao S, Olson JM, Paterson M, Yan Y, Zaja I, Liu Y, Riess ML, Kersten JR, Liang M, Wartier DC, Bosnjak ZJ, Ge ZD: MicroRNA-21 mediates isoflurane-induced

cardioprotection against ischemia-reperfusion injury via Akt/nitric oxide synthase/mitochondrial permeability transition pore pathway. *Anesthesiology* 2015; 123: 786-98

3. Ge ZD, Pravdic D, Bienengraeber M, Pratt PF Jr, Auchampach JA, Gross GJ, Kersten JR, Wartier DC: Isoflurane postconditioning protects against reperfusion injury by preventing mitochondrial permeability transition by an endothelial nitric oxide synthase-dependent mechanism. *Anesthesiology* 2010; 112: 73-85
4. Ge ZD, Ionova IA, Vladic N, Pravdic D, Hirata N, Vasquez-Vivar J, Pratt PF, Jr., Wartier DC, Pieper GM, Kersten JR: Cardiac-specific overexpression of GTP cyclohydrolase 1 restores ischaemic preconditioning during hyperglycaemia. *Cardiovasc Res* 2011; 91: 340-9
5. Gamez-Mendez AM, Vargas-Robles H, Rios A, Escalante B: Oxidative stress-dependent coronary endothelial dysfunction in obese mice. *PLoS One* 2015; 10: e0138609