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

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ABSTRACT

Background: Diabetes impairs the cardioprotective effect of volatile anesthetics, yet the mechanisms are still murky. We examined the regulatory effect of isoflurane on microRNA-21, endothelial nitric-oxide synthase, and mitochondrial respiratory complex I in type 2 diabetic mice.

Methods: Myocardial ischemia/reperfusion injury was produced in obese type 2 diabetic (db/db) and C57BL/6 control mice *ex vivo* in the presence or absence of isoflurane administered before ischemia. Cardiac microRNA-21 was quantified by real-time quantitative reverse transcriptional–polymerase chain reaction. The dimers and monomers of endothelial nitric-oxide synthase were measured by Western blot analysis. Mitochondrial nicotinamide adenine dinucleotide fluorescence was determined in Langendorff-perfused hearts.

Results: Body weight and fasting blood glucose were greater in db/db than C57BL/6 mice. Isoflurane decreased left ventricular end-diastolic pressure from 35 ± 8 mmHg in control to 23 ± 9 mmHg (P = 0.019, n = 8 mice/group, mean ± SD) and elevated ±dP/dt 2 h after post-ischemic reperfusion in C57BL/6 mice. These beneficial effects of isoflurane were lost in db/db mice. Isoflurane elevated microRNA-21 and the ratio of endothelial nitric-oxide synthase dimers/monomers and decreased mitochondrial nicotinamide adenine dinucleotide levels 5 min after ischemia in C57BL/6 but not db/db mice. MicroRNA-21 knockout blocked these favorable effects of isoflurane, whereas endothelial nitric-oxide synthase knockout had no effect on the expression of microRNA-21 but blocked the inhibitory effect of isoflurane preconditioning on nicotinamide adenine dinucleotide.

Conclusions: Failure of isoflurane cardiac preconditioning in obese type 2 diabetic db/db mice is associated with aberrant regulation of microRNA-21, endothelial nitric-oxide synthase, and mitochondrial respiratory complex I. **(ANESTHESIOLOGY 2018; 128:117-29)**

OBESITY and type 2 diabetes mellitus are main health and economic problems with a dramatic increase in prevalence and incidence globally.[1] Compared with individuals without obesity and diabetes, obese type 2 diabetic patients have a significantly higher incidence of coronary heart disease.[2] Coronary artery bypass graft surgery can help restore blood flow to an area of the heart and thus is often used to treat coronary heart disease. At present, 15 to 30% of the patients who undergo coronary artery surgery are obese and diabetic.[1,2] They have an increased mortality and poorer clinical recovery after cardiac surgery than nonobese, nondiabetic patients.[3] During coronary artery bypass graft surgery, coronary blood flow is interrupted, and the heart is put into electromechanical arrest. After the surgery has been completed, the blood flow is restored to the particular area of myocardium, causing ischemia/reperfusion injury. Attenuation or loss of cardioprotection against perioperative ischemia/reperfusion injury in obesity and diabetes can be an important and direct cause of poorer clinical outcomes.

What We Already Know about This Topic

- Diabetes impairs the cardioprotective effect of volatile anesthetics

What This Article Tells Us That Is New

- This study determined the regulatory effect of isoflurane on microRNA-21, endothelial nitric-oxide synthase, and mitochondrial respiratory complex I in type 2 diabetic mice
- Failure of isoflurane cardiac preconditioning in obese type 2 diabetic db/db mice is associated with aberrant regulation of microRNA-21, endothelial nitric-oxide synthase, and mitochondrial respiratory complex I

Volatile anesthetics, such as isoflurane and sevoflurane, are commonly used to maintain the state of general anesthesia during cardiac surgery.[4,5] Brief periods of administration of volatile anesthetics before myocardial ischemia potently reduce myocardial ischemia/reperfusion injury in human studies and in a variety of animal models of myocardial ischemia/reperfusion injury.[6] This phenomenon is similar to

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ischemic preconditioning, termed anesthetic preconditioning.[7,8] However, a study from our laboratory found that the cardioprotective effect of isoflurane was markedly attenuated in streptozotocin-induced type 1 diabetes.[9] Whether isoflurane-induced cardioprotection is attenuated in models of obesity and diabetes and the underlying mechanisms remain to be elucidated.

MicroRNAs are a class of single-stranded, non–protein-coding RNAs of approximately 22 nucleotides in length. By targeting specific coding mRNAs for degradation or translation repression, microRNA levels influence the magnitude of target gene repression.[10] MicroRNA-21 is a highly expressed microRNA in the cardiovascular system and is involved in divergent pathophysiologic processes related to ischemia/reperfusion injury and ischemic conditioning, such as apoptosis, myocardial tolerance to ischemia/reperfusion injury, cardiac growth and differentiation, inflammation, and formation of myocardial fibrosis.[11–14] The precise molecular pathways involved in the regulation of ischemia/reperfusion injury by microRNA-21 have not been fully elucidated. There is evidence that the regulation of microRNA-21 impacts the expression and function of Akt, nitric-oxide synthase, phosphatase and tensin homolog, programed cell death protein 4, and the mitochondrial permeability transition pore, which are crucial for myocardial ischemia/reperfusion injury.[11,15,16]

Recently, we demonstrated that microRNA-21 mediates isoflurane-induced cardioprotection against ischemia/reperfusion injury through favorable regulation of endothelial nitric-oxide synthase (eNOS) and suppression of the opening of the mitochondrial permeability transition pore.[16] Multiple lines of evidence suggest that microRNA-21, eNOS, and mitochondria are aberrantly regulated in diabetes and obesity.[17–19] How isoflurane regulates microRNA-21, eNOS, and mitochondria in obesity and type 2 diabetes mellitus remains unclear. In the present study, we examined the effects of isoflurane preconditioning on microRNA-21, eNOS, and mitochondrial respiratory complex I in the db/db mouse that is widely used as an animal model of type 2 diabetes mellitus with obesity.[20]

Materials and Methods

Expanded methods and results are described in the Supplemental Digital Content (http://links.lww.com/ALN/B549).

Animals

B6.BKS(D)-*Lepr*$^{db/J}$ (db/db), microRNA-21 knockout, and eNOS knockout mice on a C57BL/6 background were purchased from The Jackson Laboratory (USA). The microRNA-21 knockout mice were mated with C57BL/6 mice for seven generations at Medical College of Wisconsin. C57BL/6 wild-type mice were used as control. The experimental procedures were approved by the Animal Care and Use Committee of the Medical College of Wisconsin and conformed to the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Academy of Sciences, 8th edition, 2011).

Measurements of Blood Pressure and Fasting Blood Glucose

Male C57BL/6 and db/db mice at 12 to 14 weeks of age were fasted for 12 h. The animals were anesthetized by intraperitoneal injection of 80 mg/kg pentobarbital sodium and ventilated with room air supplemented with 100% O_2 at approximately 102 breaths/min. Body temperature was maintained between 36.8 and 37.3°C throughout the experiment by using a heating pad (model TC-1000; CWE Inc., USA). The right carotid artery was cannulated with a Millar pressure catheter (model SPR-1000; Millar Instruments, USA) inserted into the right carotid artery, as described.[18] The catheter was connected to a pressure transducer (ADInstruments, Australia) and a Powerlab data acquisition system (ADInstruments). After 30 min of stabilization, blood pressure was continuously recorded for 20 min. A thoracotomy was performed, and the left ventricle was punctured with a 27-gauge needle. Blood glucose was measured with a blood gas analyzer (ABL-725 radiometer; Radiometer America Inc., USA). After the mice were euthanized, the heart and the left ventricle were weighed. Heart weight and left ventricular weight were normalized to body weight.

Transthoracic Echocardiography

The mice were sedated by the inhalation of 1.50% isoflurane and oxygen. Noninvasive transthoracic echocardiography was performed with a VisualSonics Vevo 770 High-resolution Imaging System (Canada) equipped with a 30-MHz transducer (Scanhead RMV 707), as described previously.[21,22] Measurements of left ventricular wall thickness and the internal diameters of left ventricular chamber were made by two-dimension guided M-mode according to the American Society of Echocardiography.[23] Estimations of left ventricular volumes and ejection fraction were derived from Teichholz methods in diastole and systole.[24] Pulsed Doppler waveforms recorded in the apical four-chamber view were used for the measurements of the peak velocities of mitral early mitral inflow and late mitral inflow waves, isovolumic contraction time, ejection time, and isovolumic relaxation time of the left ventricle. Myocardial performance index was calculated with the following formula: myocardial performance index = (isovolumic contraction time + isovolumic relaxation time)/ejection time.

Myocardial Ischemia/Reperfusion Injury Ex Vivo

Langendorff Perfusion of Mouse Hearts. Mouse hearts were mounted on a Langendorff apparatus and perfused retrogradely through the aorta at a constant pressure of 80 mmHg with Krebs–Henseleit buffer at 37°C, as described.[21,25] The buffer was continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide *via* in-line filter (5-μm pore size). A fluid-filled plastic balloon was inserted into the chamber of the left ventricle *via* the mitral valve and connected

to a pressure transducer for continuous measurement of left ventricular pressure. The hearts were immersed in perfusate maintained at 37.2 ± 0.3°C, and the balloon was inflated to a diastolic pressure of ~5 to 10 mmHg. Coronary flow was monitored by an in-line flow probe connected to a flow meter (Transonics Systems Inc., USA). The signal of the left ventricle was monitored to obtain heart rate and left ventricular dP/dt. Left ventricular developed pressure (LVDP) was calculated as the difference between the systolic and end-diastolic pressure of the left ventricle. Global ischemia/reperfusion was produced by cessation of perfusion followed by reperfusion at a designated time.

Experimental Protocols. Langendorff-perfused hearts were used in the following three protocols. Protocol 1 determined the effect of isoflurane on cardiac function in C57BL/6 and db/db mice. Mouse hearts were assigned to the following four groups (n = 10 C57BL/6 or 12 db/db mouse hearts/group): control, db/db, isoflurane, and db/db+isoflurane. All hearts were stabilized for 30 min and subjected to 30 min of no-flow global ischemia followed by 2 h of reperfusion. Isoflurane was bubbled into Krebs–Henseleit solution using an agent-specific vaporizer (Ohio Medical Instruments, USA) placed in the 95% oxygen and 5% carbon dioxide gas mixture line. Isoflurane concentrations in the coronary effluent were determined by a gas chromatography. Isoflurane at 1.4% produced 0.5 mM isoflurane in the coronary effluent (approximately 1.0 maximum alveolar concentration). In the isoflurane and db/db+isoflurane groups, the hearts were perfused with 2 cycles of 5-min Krebs–Henseleit solution containing 0.5 mM isoflurane/5-min Krebs–Henseleit solution without isoflurane followed by a 10-min washout before ischemia. Heart rate, left ventricular end-diastolic pressure (LVEDP), LVDP, +dP/dt (maximum rate of LVDP increase), –dP/dt (maximum rate of LVDP decrease), and coronary flow rate at baseline, 10, 20, and 30 min after ischemia, and 10, 30, 60, 90, and 120 min after reperfusion were determined. Blinding methods were not used because db/db mice had different phenotypes from C57BL/6 control mice.

Protocol 2 studied the effect of microRNA-21 knockout on isoflurane-induced improvement in cardiac function. MicroRNA-21 knockout and C57BL/6 mouse hearts were divided into the following four groups (n = 10 hearts/group): control, microRNA-21 knockout, isoflurane, and microRNA-21 knockout+isoflurane. All hearts were stabilized for 30 min and subjected to 30 min of no-flow global ischemia followed by 2 h of reperfusion with or without 1.4% isoflurane administered before ischemia. The values of ±dP/dt were determined at baseline and 2 h after post-ischemic reperfusion.

Protocol 3 determined the importance of eNOS in isoflurane-induced cardioprotection. C57BL/6 and eNOS knockout mouse hearts were divided into the following four groups (n = 10 hearts/group): control, endothelial nitric-oxide synthase knockout, isoflurane, and endothelial nitric-oxide synthase knockout+isoflurane. All hearts were stabilized for 30 min and subjected to 30 min of no-flow global ischemia followed by 2 h of reperfusion with or without 1.4% isoflurane administered before ischemia. The values of ±dP/dt were determined at baseline and 2 h after post-ischemic reperfusion.

Quantitative Reverse Transcriptional–Polymerase Chain Reaction Analysis of MicroRNA-21

C57BL/6 and db/db mice at 12 to 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 μl using a rodent ventilator (Hugo Sachs Electronik; Harvard Apparatus, Germany). Under a dissecting microscope (Thermo Fisher Scientific Inc., USA), an 8-0 nylon suture is passed below the left main descending coronary artery 1 to 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.[25] 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 the electrocardiographic 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 and 37.5°C throughout the experiment by using a heating pad (model TC-1000). The heart was excised, and the left ventricle was homogenized at 4°C for real-time quantitative reverse transcriptional–polymerase chain reaction analysis of microRNA-21.[26]

MicroRNA-21 Extraction and Real-time Quantitative Reverse Transcriptional–Polymerase Chain Reaction Analysis. Total RNA from the left ventricle was extracted using Qiazol reagent according to the protocol of the manufacturer (Qiagen, USA). Chloroform was added, and samples were centrifuged to facilitate phase separation. The aqueous phase was extracted and combined with ethanol in miRNeasy mini spin columns (Qiagen). Total RNA was eluted in RNase-free water. The concentration of extracted total RNA was quantified by the Epoch spectrophotometer (Biotek, USA). Samples were considered pure if the A260/280 ratio was between 1.9 and 2.0. One μg of total RNA from each sample was used to generate complementary DNA using miScript reverse transcriptase mix, nucleics mix, and HiFlex buffer (Qiagen). To analyze the microRNA-21 expression, a master mix (25 μl/well) containing the template cDNA (4.5 ng/well), RNase-free water, and miScript SYBR Green (Qiagen), and the primers (microRNA-21 or the housekeeping gene, Rnu-6) were prepared according to the manufacturer's directions. Real-time quantitative reverse transcriptional–polymerase chain reaction was conducted using the Bio-Rad iCycler real-time polymerase chain reaction detection system. Real-time

quantitative reverse transcriptional–polymerase chain reaction for each sample was run in triplicate. Expression of microRNA-21 was normalized by expression of Rnu-6. The relative gene expressions were calculated in accordance with the ΔΔCt method. Relative microRNA levels were expressed as percentages compared to non–isoflurane-exposed controls.

Immunoblotting

Pentobarbital-anesthetized C57BL/6 and db/db mice at 12 to 14 weeks of age were subjected to 30 min of coronary artery occlusion followed by 2 h of reperfusion *in vivo* with or without 1.4% isoflurane administered before coronary artery occlusion. The myocardium from the area at risk of mouse hearts was harvested and homogenized in a buffer containing 20.0 mM 3-(*N*-morpholino)propanesulfonic acid, 2.0 mM EGTA, 5.0 mM EDTA, protease inhibitor cocktail (1:100; Calbiochem, USA), phosphatase inhibitors cocktail (1:100; Calbiochem), 0.5% detergent (Nonidet P-40 detergent, pH 7.4; Sigma–Aldrich, USA). Immunoblots were performed using standard techniques, as described.[27] Briefly, nonboiled protein lysate that contained 50 μg of protein was resolved by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 4°C overnight.[26] Membranes were incubated with a 1:2,000 dilution of mouse anti-eNOS monoclonal antibodies (BD Transduction Laboratories, USA). The membrane was washed and then incubated with the appropriate anti-mouse secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence followed by densitometric analysis using image acquisition and analysis software (ImageJ; National Institutes of Health, USA).

Measurements of Mitochondrial Nicotinamide Adenine Dinucleotide Levels in Langendorff-perfused Hearts

Mitochondrial nicotinamide adenine dinucleotide (reduced form [NADH]) fluorescence in mouse hearts was determined in a Langendorff apparatus placed within a light-proof Faraday cage to block the light, as described.[16] A fiberoptic cable was placed against the left ventricle of Langendorff-prepared mouse hearts to excite and record transmyocardial fluorescence at a wavelength of 456 nm during ischemia and reperfusion. The two proximal ends of the fiberoptic cable were connected to a modified spectrophotofluorometer (Photon Technology International, Canada). Fluorescence was excited with light at the appropriate wavelength (λ) from a xenon arc lamp at 75 W filtered through a monochromator (Delta RAM; Photon Technology International, USA). NADH signal was recorded continuously using a Powerlab data acquisition system (ADInstruments) at baseline and during ischemia and reperfusion.

Statistical Analysis

All data are expressed as means ± SD. Two-way repeated measures analysis of variance test was used to evaluate the differences in body weight, mean arterial blood pressure, heart weight, and the ratio of heart/body weight, and echocardiographic data. One-way analysis of variance followed by Bonferroni *post hoc* test was used to analyze LVEDP, LVDP, ±dP/dt, expression of microRNA-21, or the ratio of eNOS dimers/monomers. Repeated-measures analysis of variance followed by Bonferroni multiple comparison test was used to evaluate differences in NADH fluorescence. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., USA). A value of P less than 0.05 (two-tailed) was considered statistically significant.

In *ex 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. The value of +dP/dt in C57BL/6 mice 2 h after reperfusion without the treatment of isoflurane is typically 600 ± 120 mmHg/s, and +dP/dt in C57BL/6 mice treated with 1.4% isoflurane is around 800 ± 160 mmHg/s.[16] Based upon an average SD of 120 from our prior work with ischemia/reperfusion injury in C57BL/6 mice,[21] n = 8/group will allow for detection of a difference between groups of up to 240 at $P < 0.05$. Thus, 8 C57BL/6 or db/db mice per group were needed for these experiments.

Results

Characteristics of db/db Mice

db/db mice at 12 to 14 weeks of age displayed obesity and had an increased body weight compared with C57BL/6 mice (48.4 ± 3.4 g in db/db and 27.4 ± 2.4 g in C57BL/6 group, $P < 0.0001$, n = 10 mice/group; fig. S1, Supplemental Digital Content, http://links.lww.com/ALN/B549). Blood glucose was higher in db/db than C57BL/6 mice (380 ± 59 mg/dl in db/db and 99 ± 31 mg/dl in C57BL/6 group, $P < 0.0001$, n = 10 mice/group). Mean arterial blood pressure was comparable between db/db and C57BL/6 mice (114 ± 35 mmHg in db/db and 91 ± 33 mmHg in C57BL/6 group, n = 8 mice in db/db and 10 mice in C57BL/6 group, $P = 0.187$). The ratios of heart weight/body weight and left ventricular weight/body weight were smaller in db/db than C57BL/6 mice (heart weight/body weight: 0.0041 ± 0.0004 in db/db and 0.0052 ± 0.0006 in C57BL/6 group, $P = 0.005$, n = 10 mice/group; left ventricular weight/body weight: 0.0035 ± 0.0003 in db/db and 0.0043 ± 0.0006 in C57BL/6 group, $P = 0.001$, n = 10 mice/group).

Table 1 lists echocardiographic parameters of db/db and C57BL/6 mice. There were no significant differences between C57BL/6 and db/db mice in multiple parameters ($P > 0.05$), including heart rate, the thickness of left ventricular anterior wall and posterior wall, left ventricular end-diastolic volume, the ratio of peak mitral early mitral inflow/late mitral inflow, isovolumic contraction time of the left ventricle, and myocardial performance index. Compared with C57BL/6 mice, the ejection fraction was decreased ($P = 0.039$, n = 10 mice/

Table 1. Echocardiographic Parameters of C57BL/6 and db/db Mice

	C57BL/6	db/db
Heart rate, beats/min	458 ± 83	442 ± 69
Anterior wall at end diastole, mm	0.78 ± 0.12	0.82 ± 0.19
Anterior wall at end systole, mm	1.20 ± 0.26	1.22 ± 0.31
Posterior wall at end diastole, mm	0.79 ± 0.16	0.87 ± 0.22
Posterior wall at end systole, mm	1.20 ± 0.25	1.32 ± 0.19
Left ventricular end, μl		
Diastolic volume	59 ± 22	56 ± 18
Systolic volume	13 ± 6	28 ± 16*
Ejection fraction, %	71 ± 22	52 ± 14*
Peak mitral E, cm/s	83 ± 15	75 ± 11
Peak E/A ratio	1.71 ± 0.38	1.49 ± 0.10
Isovolumic contraction time, millisecond	13.1 ± 4.1	13.7 ± 3.9
Ejection time, ms	42.6 ± 3.7	47.0 ± 4.7*
Isovolumic relaxation time, ms	16.6 ± 2.1	18.7 ± 1.9*
Myocardial performance index	0.70 ± 0.13	0.69 ± 0.09

Ejection fraction was calculated from the formula: Ejection fraction = (left ventricular end-diastolic volume – left ventricular end-systolic volume)/left ventricular end-diastolic volume × 100.

*P < 0.05 *versus* C57BL/6 mice (mean ± SD, n = 10 mice/group).

A = late mitral inflow; E = early mitral inflow.

group), whereas left ventricular end-systolic volume, ejection time, and isovolumic relaxation time of the left ventricle were increased in db/db mice (P = 0.020, 0.032, and 0.032, n = 10 mice/group). These results suggest that the systolic and diastolic functions of the left ventricle may be impaired in db/db mice.

Isoflurane Preconditioning Improved Cardiac Function during Reperfusion in C57BL/6 but not db/db Mice

Figure 1 demonstrates cardiac function of Langendorff-perfused db/db and C57BL/6 mouse hearts subjected to ischemia/reperfusion injury in the presence or absence of isoflurane preconditioning. LVEDP at baseline was comparable among four groups (P > 0.05; fig. 1A), whereas the values of LVDP and ±dP/dt at baseline were smaller in db/db and db/db+isoflurane than control groups (P = 0.031 in LVDP, 0.009 in +dP/dt, and 0.033 in –dP/dt between db/db and control groups; P = 0.034 in LVDP, 0.024 in +dP/dt, and 0.028 in –dP/dt between db/db+isoflurane and control groups; n = 8 mice/group; figs. 1B–D). Global ischemia for 30 min resulted in the cessation of the contraction and relaxation of the hearts and an increase in LVEDP. With reperfusion, contraction and relaxation were gradually restored in all mouse hearts. There were no significant differences in LVEDP between db/db and control groups during ischemia and reperfusion (P > 0.05; fig. 1A). Compared with control groups, LVEDP was significantly decreased in isoflurane groups 1 to 2 h (35 ± 8 mmHg in control *vs.* 23 ± 9 mmHg in isoflurane, P = 0.019, n = 8 mice/group) after reperfusion but not in db/db+isoflurane groups. The values of LVDP and ±dP/dt were significantly smaller in db/db than control groups from 30 min to 2 h after reperfusion (P < 0.05; fig. 1, B–D). Compared with control groups, the values of LVDP and ±dP/dt were significantly increased in isoflurane groups from 30 min to 2 h after reperfusion and decreased in db/db+isoflurane groups (fig. 1, B–D). There were not significant differences between db/db+isoflurane and db/db groups in the values of ±dP/dt.

Failure of Isoflurane Preconditioning to Up-regulate MicroRNA-21 in db/db Mice Contributed to Attenuation of Cardioprotection

Figure 2A shows the regulation of cardiac microRNA-21 by isoflurane in C57BL/6 and db/db mice. Compared with control group, the expression of microRNA-21 gene was significantly decreased in db/db mice (P = 0.013, n = 8 mice/group). Isoflurane increased microRNA-21 by 127 ± 106% (P = 0.011, n = 8 mice) in C57BL/6 mice. In contrast, microRNA-21 expression was decreased by 27 ± 33% (n = 8 mice) by isoflurane in db/db mice (P = 0.021 between db/db+isoflurane and db/db group).

In Langendorff-perfused hearts, we examined whether microRNA-21 knockout impacted isoflurane preconditioning-elicited cardioprotection against ischemia/reperfusion injury. The values of ±dP/dt at baseline were comparable among four groups (P > 0.05, n = 7 to 9 mice/group; fig. 2, B and C). Compared with control groups, the values of ±dP/dt were significantly decreased in microRNA-21 knockout groups (P = 0.005 in +dP/dt and 0.019 in –dP/dt, n = 9 mice in control and 8 mice in microRNA-21 knockout group) and increased in isoflurane groups 2 h after postischemic reperfusion (P = 0.005 in dP/dt and 0.014 in –dP/dt, n = 9 mice/group). Interestingly, isoflurane failed to elevate ±dP/dt in microRNA-21 knockout mice 2 h after reperfusion (P = 0.141 in +dP/dt and 0.107 in –dP/dt between microRNA-21 knockout+isoflurane and control groups; P = 0.125 in +dP/dt and 0.143 in –dP/dt between microRNA-21 knockout+isoflurane and microRNA-21 knockout groups; n = 7 mice in microRNA-21 knockout+isoflurane group, 9 mice in control, and 8 mice in microRNA-21 knockout group).

Failure of Isoflurane Preconditioning to Increase eNOS Dimerization in db/db Mice Was Associated with Reduction of Cardioprotection

Figures 3, A and B show the effect of isoflurane preconditioning on eNOS dimers (230 kDa; fig. S5, Supplemental Digital Content, http://links.lww.com/ALN/B549) and monomers (130 kDa) in the myocardium of C57BL/6 and db/db mice subjected to ischemia/reperfusion injury. Compared with control groups, the ratio of eNOS dimers/monomers was decreased in db/db groups (P = 0.006, n = 5 mice/group). Isoflurane significantly increased the ratio of eNOS dimers/monomers in C57BL/6 (P = 0.002 between isoflurane and control groups, n = 5 mice/group) but not db/db mice subjected to ischemia/reperfusion injury (P = 0.506

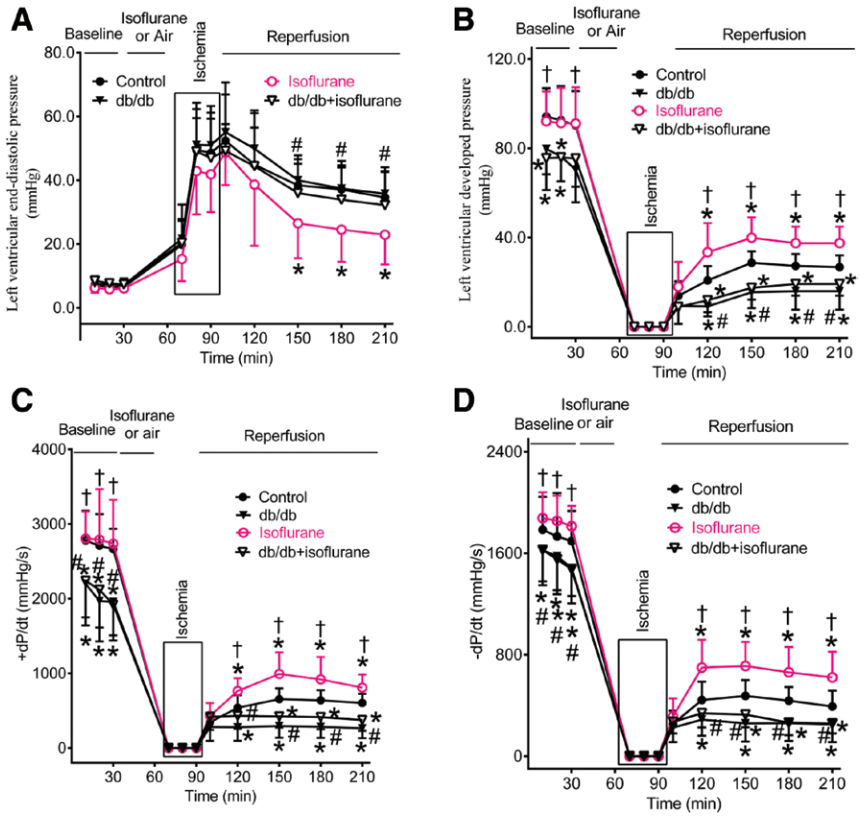

Fig. 1. Isoflurane preconditioning improved the recovery of cardiac fnction during postischemic reperfusion in Langendorff-perfused C57BL/6 mouse hearts but not db/db mouse hearts. (*A*) Left ventricular end-diastolic pressure (mean ± SD). (*B*) Left ventricular developed pressure. (*C*) The maximum rate of developed pressure rise (+dp/dt). (D) The maximum rate of developed presure decreases (–dP/dt). Control = C57BL/6 mouse hearts undergoing ischemia/reperfusion injury: db/db = db/db mouse hearts undergoing ischemia/reperfusion injury; isoflurane, C57BL/6 mouse hearts treated with 1.4% isoflurane before ischemia; db/db+isoflurane = db/db mouse hearts treated with 1.4% isoflurane before ischemia. **P* < 0.05 *versus* control groups; †*P* < 0.05 *versus* db/db groups; #*P* < 0.05 *versus* isoflurane groups (n = 8 mice/group).

between db/db+isoflurane and control groups; *P* = 0.057 between db/db+isoflurane and db/db groups; n = 5 mice/group). There were significant decreases in the ratio of eNOS dimers/monomers in db/db+isoflurane group compared with isoflurane group (*P* = 0.002, n = 5 mice/group).

To study whether eNOS is associated with isoflurane preconditioning, we examined the effect of eNOS knockout on isoflurane preconditioning in Langendorff-perfused hearts. The values of ±dP/dt were comparable among four groups at baseline (+dP/dt: *P* = 0.977 in endothelial nitric-oxide synthase knockout group, 0.777 in isoflurane group, and 0.418 in endothelial nitric-oxide synthase knockout+isoflurane group *vs.* control group; –dP/dt: *P* = 0.751 in endothelial nitric-oxide synthase knockout group, 0.358 in isoflurane group, and 0.452 in endothelial nitric-oxide synthase knockout+ soflurane group *vs.* control; n = 9 in control, 9 in endothelial nitric oxide knockout, 8 in isoflurane, and 8 hearts in endothelial nitric-oxide synthase knockout+isoflurane groups; fig. 3, C and D). There were no significant differences in the values of ±dP/dt between endothelial nitric-oxide synthase knockout and control groups. Compared with control groups, the values of ±dP/dt were significantly increased in isoflurane groups (*P* = 0.007 in +dP/dt and 0.003 in –dP/dt, n = 9 hearts in control and 8 hearts in isoflurane groups) but not in endothelial nitric-oxide synthase knockout+isoflurane groups (*P* = 0.656 in +dp/dt and 0.956 in –dP/dt, n = 9 hearts in control and 8 hearts in endothelial nitric-oxide synthase knockout+isoflurane groups) 2 h after postischemic reperfusion (fig. 3, C and D). There were significant decreases in the values of ±dP/dt in endothelial nitric-oxide synthase knockout+isoflurane groups compared with isoflurane groups (*P* = 0.005 in ±dP/dt, n = 8 hearts/group; fig. 3, C and D).

Reduction of Mitochondrial NADH Levels by Isoflurane Preconditioning in Ischemia in C57BL/6 Mice and Aberrant Regulation of NADH in db/db Mice

Mitochondrial NADH levels from Langendorff-perfused hearts at baseline were higher in db/db and db/db+isoflurane groups than control groups (*P* = 0.0006 in db/db and 0.0005 in db/db+isoflurane group *vs.* control group, n = 8 hearts in db/db and db/db+isoflurane groups and 9 hearts in control groups; fig. 4). During ischemia, the NADH signal initially increased and peaked 5 min after ischemia followed by a gradual decline in control groups. NADH fluorescence was

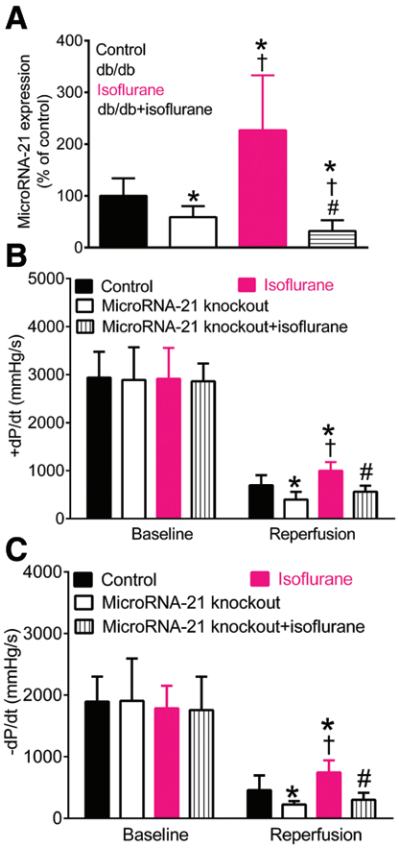

Fig. 2. Isoflurane preconditioning elevated the expression of microRNA-21 associated with cardioprotective effect in C57BL/6 mice but decreased microRNA-21 in db/db mice. (*A*) Alterations in microRNA-21 by isoflurane in C57BL/6 and db/db mice (mean ± SD, n = 8 mice/group). (*B*) Isoflurane improved +dP/dt (the maximum rate of left ventricular developed pressure increase) in C57BL/6 but not microRNA-21 knockout mice 2 h after reperfusion (reperfusion; n = 9 mice in control, 8 mice in microRNA-21 knockout group, 9 mice in isoflurane group, and 7 mice inmicroRNA-21 knockout+isoflurane group). (*C*) Isoflurane improved the values of –dP/dt (the maximum rate of left ventricular developed pressure decrease) in C57BL/6 but not microRNA-21 knockout mice 2 h after reperfusion (n = 9 mice in control, 8 mice in microRNA-21 knockout group, 9 mice in isoflurane group, and 7 mice inmicroRNA-21 knockout+isoflurane group). Control = C57BL/6 mouse hearts undergoing ischemia/reperfusion injury: db/db = db/db mouse hearts undergoing ischemia/reperfusion injury; microRNA-21 knockout = microRNA-21 knockout mouse hearts subjected to ischemia/reperfusion injury; isoflurane = C57BL/6 mouse hearts treated with 1.4% isoflurane before ischemia; db/db+isoflurane, = C57BL/6 mouse hearts treated with isoflurane before ischemia; microRNA-21 knockout+isoflurane = microRNA-21 knockout mouse hearts treated with isoflurane before ischemia. **P* < 0.05 *versus* control groups; †*P* < 0.05 *versus* db/db or microRNA-21 knockout groups; #*P* < 0.05 *versus* isoflurane groups.

significantly lower in isoflurane group than in control group 3 to 5 min after ischemia and greater in either db/db or db/db+isoflurane groups during a period of 30 min than in control group ($P < 0.05$, n = 8 to 9 hearts/group). At all time points, no significant differences existed between the db/db+isoflurabe and db/db groups ($P > 0.05$, n = 8 to 9 hearts/group). During reperfusion, the NADH signal remained relatively stable in the four experimental groups. NADH levels were higher in either db/db or db/db+isoflurane group than in control groups during a 2-h period of reperfusion.

Genetic Disruption of MicroRNA-21 Blocked the Regulation of Mitochondrial NADH by Isoflurane Preconditioning via eNOS

Figure 5 (A–C) shows the effects of microRNA-21 knockout on eNOS dimers and monomers and mitochondrial NADH levels in isoflurane preconditioned hearts 5 min after ischemia. The ratio of eNOS dimers/monomers was comparable between microRNA-21 knockout and control groups ($P = 0.224$, n = 5 hearts/group; fig. 5, A and B). Compared with control group, the ratio of eNOS dimers/monomers was significantly increased in isoflurane group ($P = 0.007$, n = 5 hearts/group) but not microRNA-21 knockout+isoflurane groups (fig. 5, A and B). There were significant differences in the ratio of eNOS dimers/monomers between microRNA-21 knockout+isoflurane and isoflurane groups ($P = 0.006$, n = 5 hearts/group). Mitochondrial NADH levels were comparable among four groups at baseline and were elevated in early ischemia (fig. 5C). Compared with the control group, mitochondrial NADH levels were elevated in the microRNA-21 knockout group ($P = 0.008$, n = 9 hearts/group) and decreased in isoflurane group 5 min after postischemic reperfusion ($P = 0.008$, n = 9 hearts/group; fig. 5C). There were significant differences in NADH levels between the microRNA-21 knockout+isoflurane and isoflurane groups. These results suggest that eNOS and mitochondrial respiratory complex I are the targets of microRNA-21 in isoflurane-induced cardioprotection.

Figure 5D shows the effect of eNOS knockout on mitochondrial NADH levels in isoflurane preconditioned hearts 5 min after ischemia. Genetic disruption of eNOS did not change the expression of cardiac microRNA-21 in the presence and absence of isoflurane preconditioning (data not shown). Mitochondrial NADH levels were comparable among four groups at baseline ($P = 0.876$ in endothelial nitric-oxide synthase knockout, 0.916 in isoflurane, and 0.709 in endothelial nitric-oxide synthase knockout+isoflurane group *vs.* control groups, n = 9 hearts in endothelial nitric-oxide synthase knockout and isoflurane groups, 8 hearts in endothelial nitric-oxide synthase knockout+isoflurane, and 10 hearts in control groups). Compared with the control group, mitochondrial NADH levels were significantly elevated in eNOS knockout groups ($P = 0.005$, n = 10 hearts in control group and 9 hearts in endothelial nitric-oxide synthase knockout group) and decreased in isoflurane group 5 min after ischemia ($P = 0.008$, n = 10 hearts in control and 9 hearts isoflurane group). There were significant differences in NADH levels between eNOS knockout and eNOS knockout+isoflurane groups 5 min after ischemia. These results suggest that

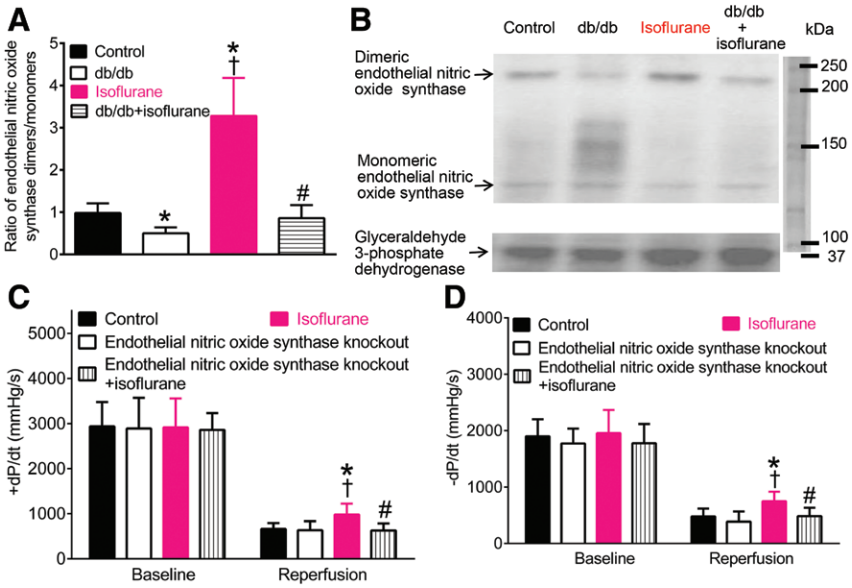

Fig. 3. Isoflurane preconditioning increased the dimerization of endothelial nitric-oxide synthase associated with cardioprotective effect in C57BL/6 but not db/db mice. (*A*) Alterations in the ratio of endothelial nitric-oxide synthase dimers/monomers by isoflurane preconditioning (mean ± SD, n = 5 mice/group). (*B*) Western blot bands showing the expression of endothelial nitric-oxide synthase dimers and monomers and glyceraldehyde-3-phosphate dehydrogenase as a loading control in mouse hearts (n = 3 hearts/blot). (*C*) Isoflurane preconditioning increased +dP/dt in C57BL/6 but not endothelial nitric-oxide synthase knockout mice 2 h after postischemic reperfusion (reperfusion; n = 9 mice in control and endothelial nitric-oxide synthase knockout groups and 8 mice in isoflurane and endothelial nitric-oxide synthase knockout+isoflurane groups). (*D*) Isoflurane preconditioning elevated the value of –dP/dt in C57BL/6 but not endothelial nitric-oxide synthase knockout mice 2 h after reperfusion (reperfusion; n = 9 mice in control and endothelial nitric-oxide synthase knockout groups and 8 mice in isoflurane and endothelial nitric-oxide synthase knockout+isoflurane groups). Control = C57BL/5 mice subjected to ischemia/reperfusion injury; db/db = db/db mice undergoing ischemia/reperfusion injury; endothelial nitric-oxide synthase knockout, endothelial nitric-oxide synthase knockout mice undergoing ischemia/reperfusion injury; isoflurane = C57BL/6 mice undergoing ischemia/reperfusion injury; db/db+isoflurane = db/db mice treated with 1.4% isoflurane before ischemia/reperfusion injury; endothelial nitric-oxide synthase knockout+isoflurane = endothelial nitric-oxide synthase knockout mice treated with isoflurane before ischemia/reperfusion injury. **P* < 0.05 *versus* control groups; †*P* < 0.05 *versus* db/db or endothelial nitric-oxide synthase knockout groups; #*P* < 0.05 *versus* isoflurane groups.

mitochondrial respiratory complex I is the target of eNOS and that microRNA-21 is not the target of eNOS in isoflurane-induced cardioprotection against ischemia/reperfusion injury.

Exclusion Criteria

Landgendorff-perfused heart experiments used 60 C57BL/6, 40 db/db, 20 microRNA-21 knockout, and 20 eNOS knockout mice. Eight C57BL/6, eight db/db, five microRNA-21 knockout, and three eNOS knockout mouse hearts were excluded from datum analysis due to any of the following undesirable situations: (1) time delay in aortic cannulation (more than 3 min), (2) aortic damage during the cannulation process, and (3) sustained arrhythmia during the 30 min of the stabilization period.

Discussion

The results of the present study demonstrate that isoflurane preconditioning fails to protect obese type 2 diabetic hearts against ischemia/reperfusion injury and causes aberrant regulation of microRNA-21, eNOS, and mitochondrial NADH in obese type 2 diabetic mice. In C57BL/6 wild-type mice subjected to ischemia/reperfusion injury, isoflurane preconditioning not only reduces myocardial infarct size (figs. S2 and S3, Supplemental Digital Content, http://links.lww.com/ALN/B549) but also improves the recovery of cardiac function during postischemic reperfusion. The favorable effects of isoflurane are attributed to increases in cardiac microRNA-21 and eNOS dimerization and decreases in mitochondrial NADH levels during early ischemia, as supported by our previous studies.[16,28] However, isoflurane preconditioning fails to produce these beneficial effects in db/db mice. These results suggest that abnormal regulation of microRNA-21, eNOS, and mitochondrial complex I might play important roles in the diminished cardioprotective effect of isoflurane preconditioning in obesity and type 2 diabetes mellitus.

The db/db mouse at 12 to 14 weeks of age had increased body weight and blood glucose. Left ventricular systolic (ejection fraction) function was significantly depressed, and left ventricular end-systolic volume and isovolumic relaxation time of the left ventricle were elevated in db/db mice. These results suggest that cardiac contractility and relaxation

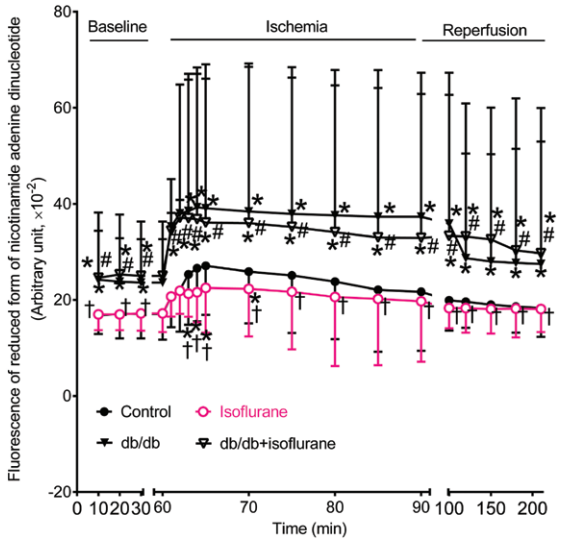

Fig. 4. Effects of diabetes and isoflurane treatment on the levels of reduced form of nicotinamide adenine dinucleotide during ischemia and reperfusion. All hearts were stabilized in Langendorff apparatus for 30 min (baseline) and perfused with the buffer with or without isoflurane before 30 min of global ischemia (ischemia) followed by 2 h of reperfusion (reperfusion). 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% isoflurane before ischemia; db/db+isoflurane = db/db mouse hearts treated with 1.4% isoflurane before ischemia. *P < 0.05 *versus* control groups; †P < 0.05 *versus* db/db groups; #P < 0.05 *versus* isoflurane groups (n = 9 mice in control, 8 mice in db/db group, 9 mice in isoflurane, and 8 mice in db/db+isoflurane group).

function are impaired in db/db mice. Because systemic blood pressure was not higher in db/db than C57BL/6 mice, cardiac dysfunction cannot be attributed to hypertension. Previous studies showed that stroke volume and heart rate were decreased in db/db mice compared with nondiabetic control mice.[20] Diabetes results in increases in atherosclerosis, arterial calcification, and vascular inflammation, leading to elevated systemic vascular resistance. It is possible that cardiac dysfunction in db/db mice may be caused by both decreased stroke volume and increased systemic vascular resistance. Clinical manifestation of coronary heart disease may present as ST-segment elevation myocardial infarction, non–ST-segment elevation myocardial infarction, or unstable angina in patients.[29] In the experiment of *in vivo* ischemia/reperfusion injury, ST segment, and T wave from electrocardiogram were normal before coronary artery occlusion (data not shown). Whether the db/db mice at 12 to 14 weeks of age had non–ST-segment elevation myocardial ischemia is not known. Diabetic cardiomyopathy refers to diabetes-associated changes in the structure and function of the myocardium that is not directly attributable to other confounding factors such as coronary heart disease or hypertension.[30] Possibly, the db/db mouse develops diabetic cardiomyopathy at 12 to 14 weeks of age.

Although clinical studies show diabetes exacerbates myocardial ischemia/reperfusion injury,[31,32] previous studies report that diabetes can reduce, increase, or have no effect on myocardial infarct size in experimental models of animals.[33,34] In the present study, the values of LVDP and ±dP/dt during postischemic reperfusion were significantly decreased in isolated hearts of db/db mice compared with age-matched C57BL/6 mice, suggesting exacerbation of ischemia/reperfusion injury in db/db mice. The db/db mouse develops obesity, hyperglycemia, atherosclerosis, insulin resistance, etc.[35] Mounting evidence suggests that cardiovascular risk factors, such as diabetes, obesity, hypertension, and atherosclerosis, among others, interact to exacerbate lethal tissue injury.[36] It is likely that interaction of these risk factors in db/db mice elevates the susceptibility of myocardium to ischemia/reperfusion injury.

In a clinical setting, the important goal of myocardial protection is to maintain cardiac function after cardiac surgery. Isoflurane preconditioning decreased LVEDP and elevated the values of LVDP and ±dP/dt during reperfusion in C57BL/6 mice. However, these favorable effects of isoflurane were lost in db/db mice. Clinical studies have shown that obese, type 2 diabetic patients undergoing cardiac surgery have poorer clinical outcome than nonobese, nondiabetic patients.[37] We believe that impaired cardioprotective effect of volatile anesthetics may contribute to increased mortality and poorer prognosis in obese type 2 diabetic patients undergoing cardiac surgery.

Mounting evidence suggests that a short period of microRNA-21 up-regulation protects the heart against ischemia/reperfusion injury and contributes to the cardioprotective effect of ischemic preconditioning.[12,14,15] Isoflurane up-regulated cardiac microRNA-21 in C57BL/6 mice subjected to ischemia/reperfusion injury, and genetic disruption of microRNA-21 abrogated the cardioprotective effect of isoflurane. These results indicate the crucial role of microRNA-21 in isoflurane-induced cardioprotection against ischemia/reperfusion injury. In contrast to C57BL/6 mice, isoflurane down-regulated cardiac microRNA-21 in db/db mice subjected to ischemia/reperfusion injury. Taken together, abnormal regulation of microRNA-21 by isoflurane in db/db mice might in part contribute to the failure of isoflurane cardiac preconditioning.

Previous studies show that up-regulation of microRNA-21 by either ischemic preconditioning or isoflurane preconditioning elevates the activity of eNOS.[15,16] In the current study, we demonstrated that isoflurane elevated the ratio of eNOS dimers/monomers in C57BL/6 but not db/db mice subjected to ischemia/reperfusion injury. The mechanisms underlying elevated eNOS dimerization by isoflurane remain elusive. It is evident that tetrahydrobiopterin bioavailability is crucial for eNOS dimerization.[38] A recent study shows that isoflurane elevated the expression of cardiac GTP cyclohydrolase 1, which is the first and rate-limiting enzyme in *de novo* biosynthesis of tetrahydrobiopterin in rats subjected

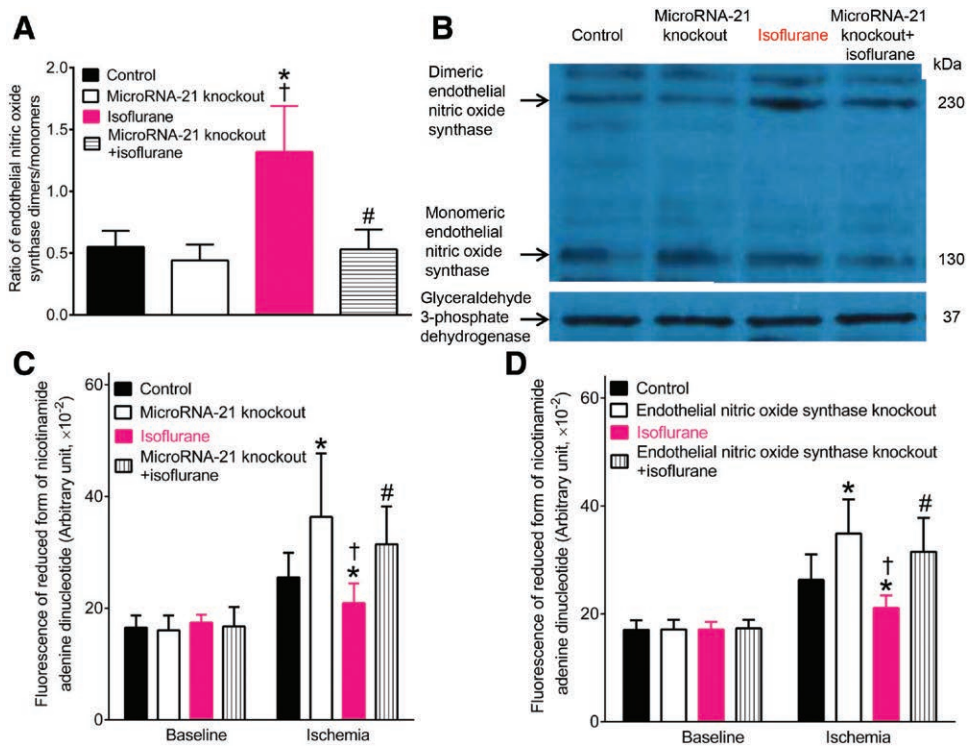

Fig. 5. MicroRNA-21 knockout blocked the regulatory effects of isoflurane preconditioning on endothelial nitric-oxide synthase and mitochondrial nicotinamide adenine dinucleotide in ischemic myocardium. (*A*) MicroRNA-21 knockout blocked isoflurane-induced increases in the ratio of endothelial nitric-oxide synthase dimers/monomers in myocardium 5 min after ischemia (mean ± SD, n = 5 hearts/group). (*B*) Representative Western blot bands showing the expression of endothelial nitric-oxide synthase dimers (230 kDa) and monomers (130 kDa) and glyceraldehyde-3-phosphate dehydrogenase (37 kDa) as a loading control in myocardium 5 min after ischemia (n = 3 hearts/blot). (*C*) MicroRNA-21 knockout blocked isoflurane-induced decreases in mitochondrial nicotinamide adenine dinucleotide levels 5 min after ischemia (n = 9 hearts in control, microRNA-21 knockout, and isoflurane groups and 8 hearts in microRNA-21 knockout+isoflurane group). All hearts were stabilized for 30 min in a Langendorff apparatus and subjected to 5 min of global ischemia. Control = C57BL/6 mouse hearts underlying 5 min of ischemia; microRNA-21 knockout, microRNA-21 knockout hearts subjected to 5 min of ischemia; isoflurane = C57BL/6 mouse hearts treated with isoflurane and subsequently underlying 5 min of ischemia; microRNA-21 knockout+isoflurane = microRNA-21 knockout hearts treated with isoflurane and subsequently subjected to 5 min of ischemia. **P* < 0.05 *versus* control groups; †*P* < 0.05 *versus* microRNA-21 knockout groups; #*P* < 0.05 *versus* isoflurane groups. (*D*) Endothelial nitric-oxide synthase knockout blocked isoflurane-induced decreases in mitochondrial nicotinamide adenine dinucleotide levels 5 min after ischemia (n = 10 hearts in control, 9 hearts in endothelial nitric-oxide synthase knockout and isoflurane groups, and 8 hearts in endothelial nitric-oxide synthase knockout+isoflurane group). Control = C57BL/6 mouse hearts underlying 5 min of ischemia; endothelial nitric-oxide synthase knockout, endothelial nitric-oxide synthase knockout hearts subjected to 5 min of ischemia; isoflurane = C57BL/6 mouse hearts treated with isoflurane and subsequently subjected to 5 min of ischemia; endothelial nitric-oxide synthase knockout+isoflurane = endothelial nitric-oxide synthase knockout hearts treated with isoflurane and subsequently subjected to 5 min of ischemia. **P* < 0.05 *versus* control groups; †*P* < 0.05 *versus* endothelial nitric-oxide synthase knockout groups; #*P* < 0.05 *versus* isoflurane groups.

to ischemia/reperfusion injury.[39] It is likely that isoflurane increases GTP cyclohydrolase 1 and tetrahydrobiopterin bioavailability, leading to eNOS dimerization. Isoflurane preconditioning decreased LVEDP and improved the values of LVDP and ±dP/dt in C57BL/6 mice 30 min to 2 h after reperfusion. These beneficial effects of isoflurane preconditioning were lost in eNOS-null mice, suggesting that eNOS is required for isoflurane preconditioning-elicited improvements in cardiac function in mice. However, isoflurane failed to elevate the ratio of eNOS dimers/monomers in db/db mice. Dimeric eNOS enzyme consists of a heme-containing oxygenase domain that binds the essential cofactor tetrahydrobiopterin, molecular oxygen, the substrate L-arginine, and a reductase domain that transfers electrons from the reduced form of nicotinamide adenine dinucleotide phosphate to flavin adenine dinucleotide and flavin mononucleotide.[40] In the presence of tetrahydrobiopterin and L-arginine, heme and oxygen reduction are coupled to the synthesis of nitric oxide.[40] Moreover, superoxide levels are increased in db/db mice.[41] It is evident that nitric oxide interacts with superoxide to form peroxynitrite, a stronger oxidant than superoxide.[42] It is likely that nitric-oxide bioavailability is decreased in db/db mice after isoflurane treatment. Previous studies have identified eNOS-derived nitric oxide as both the trigger and mediator of isoflurane preconditioning.[7] Therefore, decreased dimerization of eNOS may

be crucial for the failure of isoflurane cardiac preconditioning in db/db mice.

NADH is a substrate for the enzymatic activity of dehydrogenase that forms part of the respiratory chain and resides in the inner membrane of the mitochondria. In mitochondria, acetyl-CoA entering the citric acid cycle produces NADH and a hydroquinone form of flavin adenine dinucleotide. Mitochondrial NADH is oxidized upon donating its electrons to respiratory complex I (NADH:ubiquinone oxidoreductase) of the electron transport chain in mitochondria.[43] These electrons are sequentially relayed from complex I to ubiquinone (coenzyme 10), complex II (coenzyme Q-cytochrome *c* oxidoreductase), cytochrome *c*, and complex IV (cytochrome *c* oxidase), resulting in the formation of oxygen to water. During electron transfer, superoxide is generated, causing oxidative stress and potential induction of NF-E2-related factor 2, and activation of antioxidant response elements to the decreases in oxidative stress levels. In type 2 diabetes mellitus, high levels of glucose can induce glucose oxidation, thereby generating pyruvate and NADH, and NADH:ubiquinone oxidoreductase is inhibited.[44,45] They together cause the accumulation of NADH. Furthermore, reactive oxygen species are released from mitochondrial complex I and III.[45] Thus, the monitoring of mitochondrial NADH levels provides important information on the metabolic state of the mitochondria in terms of energy production and intracellular oxygen levels.[46] In the present study, mitochondrial NADH levels were elevated in db/db mouse hearts at baseline and in early ischemia. These results suggest that mitochondrial respiratory chain is impaired in db/db mouse hearts. It is evident that dysfunction of electron chain transportation results in the reduction of adenosine triphosphate synthesis and increases in superoxide, which contributes to exacerbation of myocardial ischemia/reperfusion injury.[47] Isoflurane significantly decreased NADH levels during early ischemia in C57BL/6 but not db/db mice. It is possible that impaired electron transfer in mitochondrial respiratory chain by diabetes also contributes to failure of isoflurane cardiac preconditioning in db/db mice.

In the present study, genetic disruption of microRNA-21 blocked isoflurane preconditioning-elicited increase in the ratio of eNOS dimers/monomers, whereas genetic disruption of eNOS did not change the regulatory effect of isoflurane on microRNA-21. Previous studies showed that up-regulation of microRNA-21 elevated the activity of eNOS, whereas microRNA-21 knockout reduced the production of the eNOS-derived nitric oxide.[48,49] Thus, in isoflurane-induced cardioprotection against ischemia/reperfusion injury, eNOS acts as an important target of microRNA-21. Genetic disruption of either microRNA-21 or eNOS blocked isoflurane preconditioning-induced decrease in mitochondrial NADH levels in early ischemia. Studies have shown that modulation of eNOS activity negatively impacts the NADH contents.[50] We speculate that isoflurane preconditioning-elicited up-regulation of microRNA-21 functionally suppresses the production of mitochondrial NADH in early ischemia through inducing eNOS dimerization (fig. 6).

In summary, the present study demonstrates that the failure of isoflurane cardiac preconditioning in db/db mice is associated with aberrant regulation of the microRNA-21, eNOS, mitochondrial respiratory complex I. The findings reveal the importance of preservation of these signaling molecules for isoflurane to protect the heart against ischemia/reperfusion injury. To find therapeutic targets for restoration of isoflurane-induced cardioprotection in obesity and diabetes, future studies will examine the effect of cardiomyocyte-specific overexpression of microRNA-21 and increased dimerization of eNOS by pharmacologic approaches (*e.g.*, tetrahydrobiopterin supplementation) on isoflurane-elicited cardioprotection against ischemia/reperfusion injury in db/db mice.

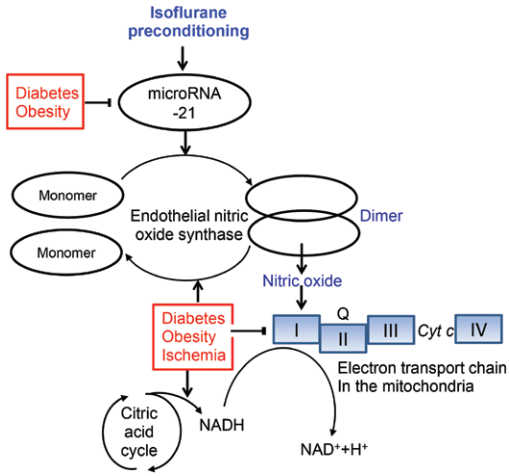

Fig. 6. Proposed mechanisms responsible for failure of isoflurane preconditioning to protect the heart against ischemia/reperfusion injury in obese type 2 diabetic mice. InC57BL/6 control mice, isoflurane preconditioning up-regulates microRNA-21, leading to the dimerization of endothelial nitric-oxide synthase through direct and/or indirect mechanisms. Dimeric endothelial nitric-oxide synthase produces nitric oxide that acts on respiratory chain complex I (reduced form of nicotinamide adenine dinucleotide [NADH]:ubiquinone) in the mitochondria and facilitates NADH to release electrons. The electrons removed from respiratory chain complex I are subsequently transferred to coenzyme Q (Q), complex III, cytochrome *c* (Cyt c), and complex IV, which uses the electrons and hydrogen ions to reduce molecular oxygen to water. Type 2 diabetes mellitus with obesity down-regulates micro RNA-21 and prevents the up-regulation of microRNA-21 by isoflurane. Moreover, diabetes, obesity, and ischemia together facilitates the transfer of endothelial nitric-oxide synthase dimers to monomers, inhibits respiratory chain complex I, and elevates the production of NADH though multiple pathways including citric acid cycle. These changes elicited by diabetes and obesity contribute to the failure of isoflurane cardiac preconditioning.

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Competing Interests

The authors declare no competing interests.

Correspondence

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