Hypoxic Preconditioning Preserves Cardiac Contractility and Reduces Infarct Size *In Vivo*

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Abstract: *Background*: Preconditioning is a powerful endogenous mechanism to protect the heart against ischemic damage. The second window of preconditioning (SWOP) is therapeutically the most attractive, but is hard to achieve by local cardiac ischemia 24 hrs before the index ischemia in the many mice models for cardiovascular pathology, because of excessive mortality during the two procedures or the period in between. Hypoxic preconditioning is an attractive alternative preconditioning stimulus. To date, the SWOP after hypoxic preconditioning has not been studied in mice concerning infarct size reduction and preservation of left ventricular contractility *in vivo*.

Aim: To determine whether transient hypoxia can induce a SWOP *in vivo* in mice.

Methods: Hypoxic preconditioning was induced by 5 cycles of 6 minutes of 6% oxygen in 24-week-old wild type mice. Twenty-four hours later, a 30 minutes coronary occlusion was performed. After 1 hour of reperfusion, *in vivo* cardiac pressure-conductance catheterization was performed with determination of the load-dependent and load-independent parameters. Infarct size was determined by TTC-staining. Sham procedures were used to obtain non-preconditioned controls.

Results: There was no mortality with the hypoxic preconditioning protocol. The left ventricular contractile parameters ejection fraction, end-systolic elastance and preload recruitable stroke work were significantly better preserved after ischemia in the preconditioned group. Diastolic relaxation (tau) was also significantly better preserved. Infarct size was reduced to half that of the non-preconditioned group.

Conclusion: Hypoxic preconditioning is a feasible stimulus to induce *in vivo* a second window of preconditioning in mice. Infarct size is reduced and cardiac contractility better preserved after 30 min regional ischemia *in vivo* by hypoxic preconditioning 24 hrs earlier.

INTRODUCTION

Preconditioning describes the phenomenon that a short episode of preceding ischemia induces improved tolerance in an organ or tissue to the deleterious effects of a subsequent longer period of ischemia [1-12]. Ischemic preconditioning is a powerful endogenous mechanism to protect the heart against ischemic damage. The second window of preconditioning (SWOP) is therapeutically the most attractive with its long standing effect from 24 hours till 72 hours. This protection can therefore be planned more readily in clinical practice and even chronically induced when ischemia is anticipated in the following days, but not exactly predictable, e.g. in patients with cardiovascular disease undergoing non–cardiac surgery.

In the past decade, numerous transgenic mouse models have been developed. They often serve as animal models to study cardiovascular diseases, for exemple heart failure, diabetes and cardiac hypertrophy. It is important to study preconditioning in these transgenic mice for 2 reasons: 1) it gives us the opportunity to investigate whether preconditioning can be induced in pathological conditions that would benefit most from its protective effects, and 2) it gives us the opportunity to further unravel the mechanism of preconditioning. Therefore a well-defined model of SWOP in mice is very welcome.

Intermittent hypoxia is an attractive method to try to induce SWOP in small animal models like mice. The method avoids cardiothoracic surgery and its inherent mortality in mouse models that are often expensive and difficult to breed. Secondly no anaesthesia, which can interfere with the preconditioning stimulus, is needed to perform hypoxic preconditioning.

In previous studies, hypoxia was shown to induce SWOP with cardiac protection against ischemic damage, but these studies all used *in vitro* techniques [13, 14].

The aim of this study is to examine whether intermittent hypoxia is capable to induce preconditioning in mice *in vivo* that is active 24 hrs later. We wanted to study infarct size and *in vivo* load-independent left ventricular contractility to accurately define the model and the preconditioning effect.

METHODS AND MATERIALS

Animal Models

Wild type mice with C57Bl6/J genetic background were purchased form Jackson Laboratories (Bar Harbour, Maine,

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USA) and housed at 22°C on a fixed 12 hour light-dark cycle. Groups were sex-matched and investigated at the age of 24 weeks. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental protocols were approved by the Institutional Animal Care Commission and Ethical Committee.

Four groups of each 6 animals were used : ischemia after preconditioning (I+P), ischemia without preconditioning (I+NP), sham procedure after preconditioning (sham+P) and sham procedure without preconditioning. (sham+NP).

Intermittent Hypoxia

Mice were placed in a Plexiglas container (Agnthos, Sweden) of 0.8 liter. A portable oxymeter (Analox O_2 EII, North Yorkshire, UK) was calibrated at 20.7% for room air and was subsequently placed in this Plexiglas container for continuous registration of the oxygen content. A mixture of 100% nitrogen and compressed air was blown through this container *via* 2 separate flowmeters. Within 30 seconds, the oxygen content was lowered to 6% oxygen. Five cycles of 6 minutes of 6% oxygen, interspersed with 6 minutes of room air were used [13]. In the non-preconditioned group, mice were placed in the plexiglas container, with only compressed air (without 100% nitrogen) blown through the container.

After this hypoxic preconditioning protocol, mice were placed in their regular cage with food and water ad libitum for 24 hours.

Coronary Occlusion

Twenty-four hours later, anesthesia was induced with urethane (1.2 g/kg) and alfa-chloralose (50 mg/kg) intraperitoneally injected. Mice were placed on a heating pad, and rectal temperature kept between 36.5 and 37.5°C. Surgery was performed under a surgical microscope. Through a midline neck incision a tracheostomy was performed and mechanical ventilation started with room air (Minivent 845; Hugo Sachs/Harvard Apparatus, March-Hugstetten, Germany).

A left anterolateral thoracotomy was performed in the 3th intercostal space. The left lower limb was positioned to the right to facilitate visualization of the left coronary artery (LAD). Two millimeter below the tip of the left auricle, a 8/0 polypropylene suture was passed under the LAD. The snare was tightened on a small plastic tubing piece of 1.5 mm in order to perform an atraumatic occlusion of the LAD. Successful coronary occlusion was visually verified by observing the distal part turning pale. Occlusion was maintained for 30 minutes. Afterwards, the snare was released, reperfusion visually checked by blushing of the area, the chest closed and a reperfusion period of 1 hour allowed before the left ventricular pressure-conductance measurements were started.

Outcome Parameters: Infarct Size Determination and *In Vivo* Left Ventricular Contractility

Towards the end of the 1 hr reperfusion period, the right carotid artery and jugular vein were prepared to allow the starting of measurements after exactly 60 min of reperfusion. A 1.4 Fr high fidelity pressure-conductance catheter (1.4-Fr, SPR-839; Millar Instruments, Houston, TX) was inserted through the right carotid artery into the left ventricle. After stabilization of the hemodynamic situation, baseline pressure-volume (PV) loops were recorded (Powerlab/4SP ADInstruments, Castle Hill, Australia) while the ventilation was momentarily turned off to avoid respiratory fluctuation of cardiac signals.

A small laparotomy was performed in the midline of the upper abdomen. The inferior caval vein was compressed between liver and diaphragm with a cotton swab, while PV-loops were recorded (occlusion loops). This was performed at least 5 times. Afterwards a 24 G catheter was introduced in the right jugular vein and parallel volume was determined by a bolus injection of 3 µl of 30% sodium chloride solution. This was performed 3 times. The pressure-conductance catheter was removed and the carotid artery ligated. The chest was reopened and the LAD was re-occluded with a 8/0 polypropylene suture at the initial occlusion site. The laparotomy was extended and the inferior caval vein was exposed infrahepatically. With a 24G needle, 0.3 ml blood was retrieved from the inferior caval vein to measure specific conductivity in 3 precalibrated cuvets.

Evans blue (0.8 ml, 1% solution) was given intravenously *via* an exchanged catheter in the right jugular vein to determine the left ventricular perfusion area at risk. After the administration of Evans blue, there was a cardiac arrest within 30 seconds. The heart was excised and placed in tissue freezing medium (Tissue-Tek, Sakura Finetek, Torrance, USA) in a – 20° C freezer for at least one hour. With a custom-made razorblade system, the heart was cut in slices of 1 mm thickness. These slices were placed for 20 minutes in triphenyl tetrazolium chloride solution (1%, 37°C, phosphate buffer pH 7.4). Afterwards they were placed for 10 minutes in paraformaldehyde (4% solution, 20 °C). All slices were weighed and photographed with a digital camera under microscopic magnification.

Data Management and Statistical Analysis

Analysis of the pressure-conductance data was performed using PVAN 3.2 software (Millar Instruments, Houston, TX). A conductance-volume calibration line was constructed with the cuvette data. All data were corrected for parallel volume and expressed in absolute volumes. Only technically acceptable loops were included in the analysis for each experiment [15].

Analysis of the morphological data was performed for each experiment. The number of pixels in each zone (total slice, risk zone, infarcted zone) was determined for all the slices of each heart with Adobe Photoshop 8.0 (Adobe System Inc.). After correction for the weight of the respective slices, the risk zone and infarcted zone were calculated for the entire heart.

Data are expressed as mean ± Standard Deviation. Differences between groups were analyzed for statistical significance by factorial ANOVA followed by a LSD post hoc test. Statistical software (Statistica 7.1, StatSoft) was used. A value of $p<0.05$ was considered significant.

RESULTS

Weight, heart weight and heart weight corrected for the tibial length were equal in the 4 groups.

Acute Effects of the Intermittent Hypoxia

All 24 mice survived the hypoxic preconditioning procedure. After initial agitation during lowering the oxygen content in the container, the mice calmed down when the oxygen content reached 6% after 30 seconds with however persistent tachypnea. After 15 seconds of exposure to room air, they demonstrated their normal behaviour pattern.

Mortality During Cardiac Ischemia and Reperfusion

In the I + NP group, 1 mouse died during the reperfusion phase after LAD occlusion. There was no mortality in the other groups.

Infarct Size (Table 1)

In both sham groups with and without the preconditioning procedure but without cardiac ischemia, there was no detectable infarct after the experimental protocol. Second window of hypoxic preconditioning was able to reduce infarct size to half that of the non-preconditioned group (49.9 ± 7.3% to 24.7 ± 7.3% of the risk zone, P<0.001).

Left Ventricular Contractility (Table 1)

The hypoxic preconditioning protocol in the sham groups not undergoing 30 min index ischemia 24 hrs later did not change left ventricular contractility parameters.

Load-Dependent Parameters

Heart rate was comparable in all groups. The risk zone of the heart was in both ischemia groups approximately 15%.

Ejection fraction, a conventional but load-dependent parameter for systolic function, was better preserved when ischemia was preceded by hypoxic preconditioning 24 hrs earlier (56.1 ± 5.4% *vs* 48.6 ± 5.9%, P=0.02).

Table 1. Phenotypic Characteristics, Infarct Size and Hemodynamic Parameters

	Group Sham+NP	Group Sham+P	Group I +NP	Group I + P
Phenotypic Characteristics				
Weight (g)	30.6 ± 5.8	27.7 ± 2.2	30.8 ± 5.9	26.4 ± 4.3
Heart weight (mg)	161.5 ± 23.4	145.3 ± 17.5	151.9 ± 16	140.3 ± 21.4
Heart weight/Tibial length (mg/cm)	79.7 ± 11.1	71.2 ± 7.6	74.6 ± 7.3	68.6 ± 9.8
Infart Size				
% Infarct of risk zone (%)	0 ± 0	0 ± 0	49.9 ± 7.3	24.7 ± 7.3 *
Risk zone of the heart (%)	0 ± 0	0 ± 0	14.4 ± 8.8	15 ± 7.4
Hemodynamic Parameters in Steady State (Load-Dependent)				
Vp (µl)	30.2 ± 2.7	32.5 ± 2.1	34.6 ± 3.2	30.2 ± 6.9
Heart rate (bpm)	576 ± 43	537 ± 55	565 ± 62	569 ± 50
Ves (µl)	11.6 ± 5.7	14.9 ± 7.5	9.9 ± 7	8.5 ± 2.5
Ved (µl)	26.1 ± 10.5	28.4 ± 13.3	15.3 ± 9.3	15.9 ± 5.3
Pes (mmHg)	67.7 ± 15	61.7 ± 13.3	54.9 ± 5.5	57.2 ± 13.7
Ped (mmHg)	2.8 ± 0.8	4.4 ± 1 #	3.6 ± 1	2.5 ± 0.7
Pmax (mmHg)	77.5 ± 13	69.4 ± 13.1	60.2 ± 6	62.6 ± 13.3
Stroke volume (µl)	17.8 ± 5.3	17.1 ± 5.5	7.5 ± 4	9.5 ± 3
Ejection fraction (%)	65.5 ± 10.6	57.7 ± 7.1	48.6 ± 5.9	56.1 ± 5.4 *
Cardiac output (µl/min)	10343 ± 3281	9210 ± 3194	4220 ± 2358	5436 ± 2041
Stroke work (mmHg*µl)	1145.6 ± 420.9	1021.3 ± 550.3	323.2 ± 193.8	405.5 ± 131.3
Ea (mmHg/µl)	4.1 ± 1.5	3.7 ± 0.5	10.5 ± 8.2	6.6 ± 2.4
dP/dt max (mmHg/s)	6648 ± 2142	5971 ± 2896	4366 ± 935	4603 ± 725
dP/dt min (mmHg/s)	-5454 ± 1882	-4803 ± 1659	- 3557 ± 882	- 4336 ± 1495
Tau G (ms)	10.7 ± 2.6	10 ± 1.6	12.5 ± 2	8.5 ± 2 *
Hemodynamic Parameters After Temporary Preload Reduction (Load-Independent)				
Ees (mmHg/µl)	8.2 ± 3	7.4 ± 1.9	6.3 ± 1.6	9 ± 1.7 *
PRSW (mmHg)	87.5 ± 5.8	84.3 ± 5.3	61.4 ± 12.3	76.6 ± 6.5 *
EDPVR (mmHg/µl)	0.2 ± 0.2	0.5 ± 0.5	0.4 ± 0.2	0.3 ± 0.2

*p < 0.05 *vs* "Ischemia without preconditioning".
#p < 0.05 *vs* "Sham procedure without preconditioning".

The other conventional load-dependent parameters (end-systolic pressure, stroke volume, cardiac output, stroke work and dP/dt_{max}) tended to be better preserved after ischemia with the preconditioning protocol, but this did not reach statistical significance.

Tau, as parameter for early diastolic relaxation was better preserved after ischemia preceded by the hypoxic preconditioning protocol (8.5 ± 2 ms *vs* 12.5 ± 2 ms, P=0.05). The other parameter for relaxation dP/dt_{min} tended to be more negative after preconditioning. No differences in left ventricular stiffness, as determined by the exponential fit of the end-diastolic pressure-volume relationship (EDPVR) could be demonstrated.

The effective arterial elastance (Ea) is defined as the ratio of the end-systolic pressure/stroke volume and is a parameter for afterload. Ea tended to be lower after preconditioning, but this didn't reach statistical significance.

Load-Independent Parameters

End-systolic elastance (Ees) is a relatively load-independent parameter which reflects the left ventricle end-systolic stiffness and is determined as the slope of the end-systolic pressure-volume relationship. Ees was better preserved after ischemia with the preconditioning protocol (9 ± 1.7 mmHg/µl *vs* 6.3 ± 1.6 mmHg/µl, P=0.04) (Fig. **1**).

Preload Recruitable Stroke Work (PRSW) is the slope of the relationship between end-diastolic volume and stroke work performed by the ventricle. Hypoxic preconditioning 24 hrs before 30 min regional cardiac ischemia led to a better preserved PRSW (76.6 ± 6.5 mmHg *vs* 61.4 ± 12.3 mmHg, P=0.03) (Fig. **2**).

DISCUSSION

Preconditioning of the heart was first described by Murry *et al*. in 1986 [1]. Since then, numerous papers have been published concerning this endogenous protection mechanism to unravel the underlying mechanisms [3]. In the past decade, numerous transgenic mouse models of cardiovascular diseases have been developed. The study of preconditioning in these models gives us the opportunity to investigate whether preconditioning can be induced in pathological conditions that would benefit most from its protective effects, and to further unravel the mechanism of preconditioning. Therefore, we wanted to characterize a feasible protocol of SWOP induction in mice.

Methods to precondition the myocardium include local ischemia, hypoxia, transient ischemia in a remote organ or pharmacologically [2-4, 8-14, 16-21]. Local ischemia is the oldest and most frequently used method to induce an early and late phase of preconditioning in mice. Guo *et al*. [16] described that a sequence of six cycles of 4 min coronary occlusion and 4 min reperfusion was protective in mice. With this technique the magnitude of protection afforded by the early phase was 75% reduction in infarct size and 48-55% by the late phase of preconditioning. Similar, this protocol was highly effective in inducing SWOP in rabbits [20-21].

The major disadvantage of regional cardiac ischemia as SWOP stimulus is the need to perform surgery to deliver it.

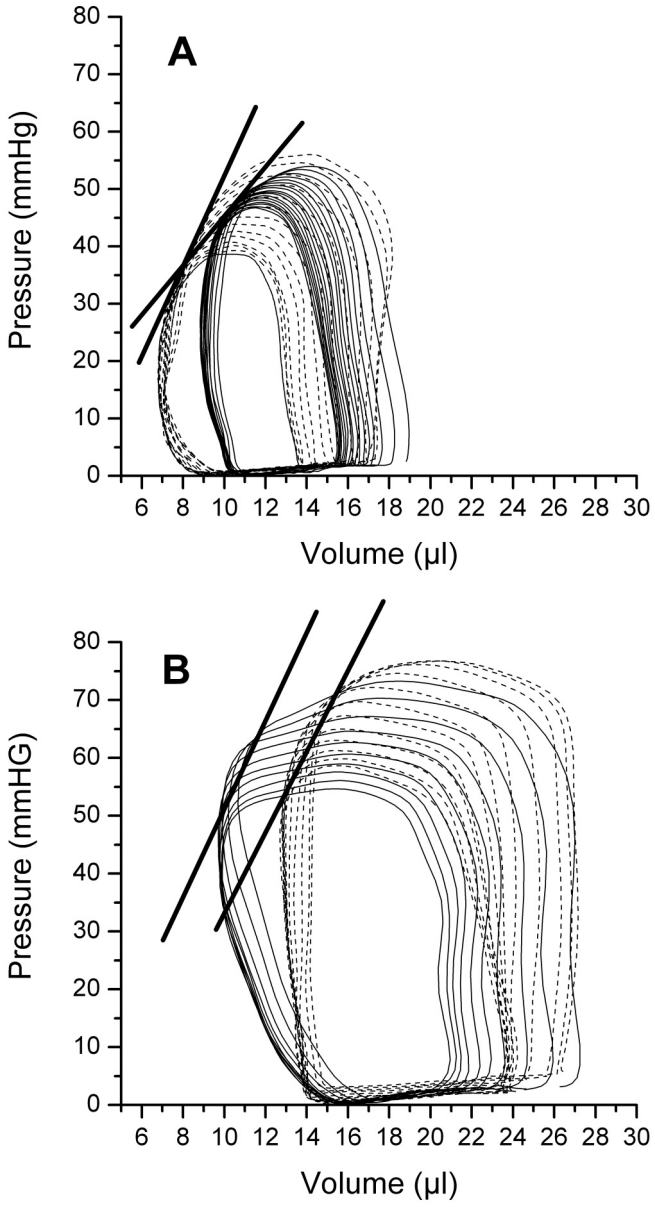

Fig. (1). (**A**) Representative example of occlusion loops and Ees-curves of the ischemia groups. Group I + P: dash loops and Group I + NP: solid loops. (**B**) Representative example of occlusion loops and Ees-curves of the sham groups. Group sham + P: dash loops and Group sham + NP: solid loops.

The required survival period between the stimulus and the index ischemia, which often requires a second cardiovascular surgery in mice, often limits the applicability. Although this is a feasible technique in lean mice, we found in a preliminary study unacceptable mortality rates of more than 50% in the obese ob/ob mouse model. Not only the limited survival after surgery to induce regional cardiac ischemia is problematic, also the fact that anesthesia is necessary for it. This anesthesia can interfere in an uncontrolled way with the preconditioning stimulus, especially if volatile anesthetics or opioid agonists are used [2, 10, 12].

Acute systemic and/or intermittent hypoxia was described in the early years 2000 as an effective preconditioning stimulus. Cai *et al*. [13] described for the first time the protocol of 5 cycles of 6 minutes, in which the oxygen con-

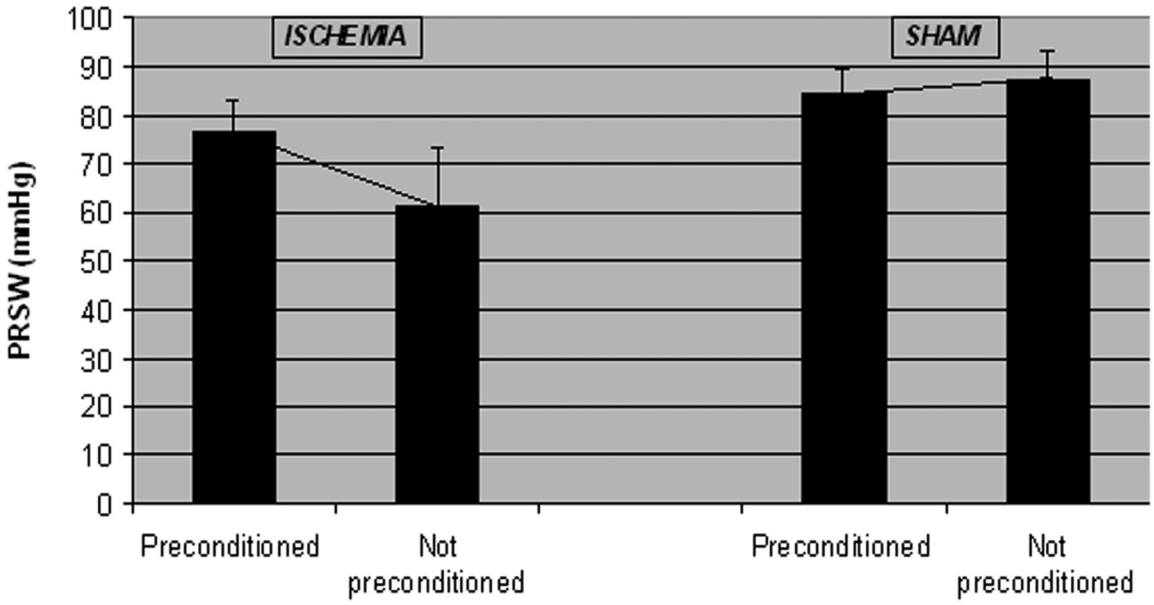

Fig. (2). PRSW is significantly (p=0.03) better preserved after the preconditioning protocol in the ischemia group. There were no statistical significant differences among the sham groups.

centration was rapidly reduced to 6%. With this method he could induce myocardial protection in mice hearts studied 24 hours later in a Langendorff system. Ischemia was induced by a perfusion stop during 30 minutes. Left ventricular developed pressure improved and infarct size was reduced [13]. With this hypoxic preconditioning protocol, no right ventricular hypertrophy and pulmonary hypertension is seen [18]. Xi *et al*. [14] studied delayed preconditioning induced by one or two cycles of 10% hypoxia for various durations (30 min, 2 h, 4 h). Infarct size was reduced only in mice pretreated with one or two cycles of 4 hour hypoxia.

To examine the *in vivo* effect of this stimulus, we performed cardiac pressure-conductance catheterization to determine left ventricular contractility with load-dependent and load-independent parameters [15, 22-24]. Infarct size measurements were used to identify the morphological damage after ischemia. In our series, there was no mortality with the described hypoxic preconditioning protocol.

Infarct size of the risk zone was significantly reduced from 49.9 ± 7.3% to 24.7 ± 7.3% after preconditioning. This reduction to about half the infarct size is comparable with the data of Guo *et al*. [16] who used local cardiac ischemia as preconditioning stimulus and the *in vitro* data of Cai *et al.* [13] with hypoxia as preconditioning stimulus.

Systolic function and active diastolic relaxation, as measured by ejection fraction and Tau were significantly better preserved. Ees and PRSW are considered as the gold standards to study ventricular contractility [24]. Accurate measurement of them requires the invasive procedure of pressure-volume measurements with different preload conditions. This parameters Ees and PRSW were respectively 42% and 25% better preserved by preconditioning after ischemia. This was never shown before.

The underlying mechanisms of the second window of preconditioning following hypoxic preconditioning in mice are not known. Cai *et al.* [13] showed that erythropoietin (EPO) mRNA expression was induced in kidneys of wild-type mice subjected to intermittent hypoxia, resulting in increased plasma EPO levels. No other data are available concerning the second window of preconditioning after hypoxic preconditioning.

In rat models, the role of NO and mitochondrial K_{ATP} channels in the hypoxic induced cardioprotection has been shown by Beguin *et al*. [25]. Rats, exposed to 4 hours of intermittent hypoxia had an infarct size reduction of 33.5% to 21.5%. This effect was abolished by L-NAME, a NOS inhibitor and the K_{ATP} channel blocker 5-HD. More recently, Beguin *et al*. [26] showed that delayed preconditioning in rats is mediated by protein kinase C and triggered by p38 MAPK and Erk1/2. Another mechanism for this protection in rats is the formation of reactive oxygen species. Kolar *et al*. [27] showed that the protective effect of chronic intermittent hypoxia in rats hearts was abolished by treatment with the antioxidant N-acetylcysteine.

In mice models following an ischemic preconditioning protocol (6 cycles of 4 min occlusion/4 min reperfusion), iNOS plays an important role. Guo *et al.* [28] showed that iNOS was increased with 40% in the ischemic/reperfused region. Our model provides a comparable degree of cardiac protection. It is thus tempting to suggest that iNOS is a potential mechanism in hypoxic preconditioning in mice.

Also COX-2 plays an important role. COX-2 activity was upregulated following ischemic preconditioning in mice, but, in contrast, the absence of iNOS prevented the activity of COX-2 protein. In the same study, it was found that iNOS and COX-2 coprecipitated 24 hours after ischemic preconditioning in the myocardium, indicating a physical interaction between these proteins. The upregulation of COX-2 protein expression after ischemic preconditioning was mediated by a JAK1/2-STAT1/3-signaling cascade [29].

In conclusion, this study shows that hypoxic preconditioning is a feasible stimulus to induce *in vivo* a second win-

dow of preconditioning in mice. Cardiac contractility can significantly be preserved by this technique. The degree of cardiac protection induced by the intermittent hypoxia protocol seems to be comparable with regional cardiac ischemia as preconditioning stimulus.

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