Original Research

Phosphorylation of phosphatidylinositol-3-kinase-protein kinase B and extracellular signal-regulated kinases 1/2 mediate reoxygenation-induced cardioprotection during hypoxia

Giuseppina Milano[1], Ludwig K von Segesser[1], Sandrine Morel[2], Ana Joncic[1], Paola Bianciardi[3], Giuseppe Vassalli[1,4] and Michele Samaja[3]

[1]Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne CH-1011; [2]Foundation for Medical Research, Division of Cardiology, University Hospital Geneva, 1211 Geneva, Switzerland; [3]Department of Medicine, Surgery and Dentistry, University of Milan, Viadi Rudini '8, I-20142 Milan, Italy; [4]Cardiology, Fondazione Cardiocentro Ticino, Lugano, Switzerland
Corresponding author: Giuseppina Milano, Department of Cardiovascular Surgery, Centre Hospitalier Universitaire Vaudois (CHUV), 46 rue du Bugnon, Lausanne CH-1011, Switzerland. Email: giuseppina.milano@chuv.ch

Abstract

In vivo exposure to chronic hypoxia (CH) depresses myocardial performance and tolerance to ischemia, but daily reoxyenation during CH (CHR) confers cardioprotection. To elucidate the underlying mechanism, we tested the role of phosphatidylinositol-3-kinase-protein kinase B (Akt) and p42/p44 extracellular signal-regulated kinases (ERK1/2), which are known to be associated with protection against ischemia/reperfusion (I/R). Male Sprague–Dawley rats were maintained for two weeks under CH (10% O_2) or CHR (as CH but with one-hour daily exposure to room air). Then, hearts were either frozen for biochemical analyses or Langendorff-perfused to determine performance (intraventricular balloon) and tolerance to 30-min global ischemia and 45-min reperfusion, assessed as recovery of performance after I/R and infarct size (tetrazolium staining). Additional hearts were perfused in the presence of 15 μmol/L LY-294002 (inhibitor of Akt), 10 μmol/L UO-126 (inhibitor of ERK1/2) or 10 μmol/L PD-98059 (less-specific inhibitor of ERK1/2) given 15 min before ischemia and throughout the first 20 min of reperfusion. Whereas total Akt and ERK1/2 were unaffected by CH and CHR *in vivo*, in CHR hearts the phosphorylation of both proteins was higher than in CH hearts. This was accompanied by better performance after I/R (heart rate $\times$ developed pressure), lower end-diastolic pressure and reduced infarct size. Whereas the treatment with LY-294002 decreased the phosphorylation of Akt only, the treatment with UO-126 decreased ERK1/2, and that with PD-98059 decreased both Akt and ERK1/2. In all cases, the cardioprotective effect led by CHR was lost. In conclusion, *in vivo* daily reoxygenation during CH enhances Akt and ERK1/2 signaling. This response was accompanied by a complex phenotype consisting in improved resistance to stress, better myocardial performance and lower infarct size after I/R. Selective inhibition of Akt and ERK1/2 phosphorylation abolishes the beneficial effects of the reoxygenation. Therefore, Akt and ERK1/2 have an important role to mediate cardioprotection by reoxygenation during CH *in vivo*.

Keywords: chronic hypoxia, reoxygenation, Langendorff perfusion, LY-294002, PD-98059, UO-126, ischemia/reperfusion, hypoxic preconditioning

Experimental Biology and Medicine 2010; **235**: 401–410. DOI: 10.1258/ebm.2009.009153

Introduction

A pathophysiological feature in several diseases, chronic hypoxia (CH) represents a major challenge for myocardial performance and resistance to ischemia and reperfusion (I/R). When rats are exposed to 15-day CH, their hearts exhibit impaired tolerance to oxygenation as compared with normoxic animals (N).[1] However, if hypoxic animals are exposed to room air for one hour/day, or CH with animal aeration (CHR), operations that reoxygenate the heart, both myocardial performance and tolerance to oxygenation improve markedly.[1] Thus, although differing only for daily one-hour reoxygenation episodes, CH and CHR elicit opposed responses in terms of cardioprotection. These responses are accompanied by differential regulation of sarcK^+_{ATP} and mitoK^+_{ATP} channels,[2] mitogen-activated protein kinases (MAPK),[3] hypoxia-inducible factor-1α (HIF),[4] apoptosis[4] and nitric oxide (NO),[1] but not myocardial morphology.[5]

The molecular mechanisms leading to CHR-induced cardioprotection are unknown, but it is tempting to speculate that they share some common signaling paths with the

ISSN: 1535-3702

processes underlying ischemic preconditioning (IP). The IP paradigm originates from the observation that repetitive sublethal ischemic insults finally make hearts more resistant to severe ischemia. IP might explicate its effect through an early (minutes) and a late (hours to days) window. The latter window of protection likely involves persisting protein and/or structural changes. Two cell survival signaling pathways, phosphatidylinositol-3-OH kinase-protein kinase B (Akt) and p42/p44 extracellular signal-regulated kinases (ERK1/2) are known to play a crucial role.[6] In the perspective of clarifying the relationship of CHR with cardioprotection, in this study we examine the impact of Akt and ERK1/2 activation as protective factors in a protocol whereby hearts from CHR animals are exposed to I/R. As CHR is applied throughout a two-week period, such protocol closely resembles that elicited by late IP, or the so-called second window of protection.

Materials and methods

Animals

The experimental design of this study is shown in Figure 1. Male Sprague–Dawley rats ($n = 82$), weighting 245 ± 1 g at entry into the study, were exposed to either CH (breathing a normobaric gas at 10% O_2, 90% N_2 for 15 days, $n = 19$) or CHR (as CH, but exposed to room air for 1 h/day, $n = 47$); N ($n = 16$) were the control. Rats had free access to water and a laboratory diet until 24 h before sacrifice. CH and CHR rats were anesthetized (10 mg Na-thiopental/100 g and 1500 IU heparin, intraperitoneally) in a compensation chamber kept at 10% O_2. CHR rats were anesthetized 24 h after the 15th daily reoxygenation event. The investigation conforms to 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) and with Swiss law and local ethical committee guidelines for animal research.

After anesthesia, some animals ($n = 6$, 6 and 5, for N, CH and CHR, respectively) were sacrificed immediately, blood was withdrawn and hearts quickly removed, rinsed in ice-cold phosphate-buffered saline (PBS) (pH 7.4), clamped between steel tongs precooled with liquid nitrogen and stored at -80°C for biochemical analyses. The other hearts ($n = 10$, 13 and 42, respectively) were used for the Langendorff perfusion.

Biochemical tests

Blood

Immediately after heart removal, a blood sample was withdrawn into a heparinized tube and divided into two aliquots. One was used for hemoglobin concentration, hematocrit and red blood cell count (Abbott Cell-dyn 3500 R System, Baar, Switzerland). The other was centrifuged (2500 rpm for 10 min); the plasma was frozen and used to measure nitrates + nitrites by the Griess reaction.[7] After conversion of nitrates into nitrites by nitrate reductase, nitrites were reacted with 0.1% naphthyl ethylene diamine that yields a colored dye monitored by spectrophotometry at $\lambda = 540$ nm. The concentration of nitrates + nitrites was calculated against a calibration curve.

Extracellular signal-regulated kinases 1/2, phosphatidylinositol-3-kinase-protein kinase B and p38 mitogen-activated protein kinase

Frozen tissue was homogenized in ice with a Polytron in 1 mL radioimmunoprecipitation assay (RIPA) buffer (1 mL RIPA buffer 1 ×, 0.1% phenylmethanesulphonylfluoride (PMSF), 0.15% protease inhibitor cocktail solution and 0.1% sodium orthovanadate). Subsequently, samples were centrifuged at 12,000 rpm for 15 min at 4°C. The

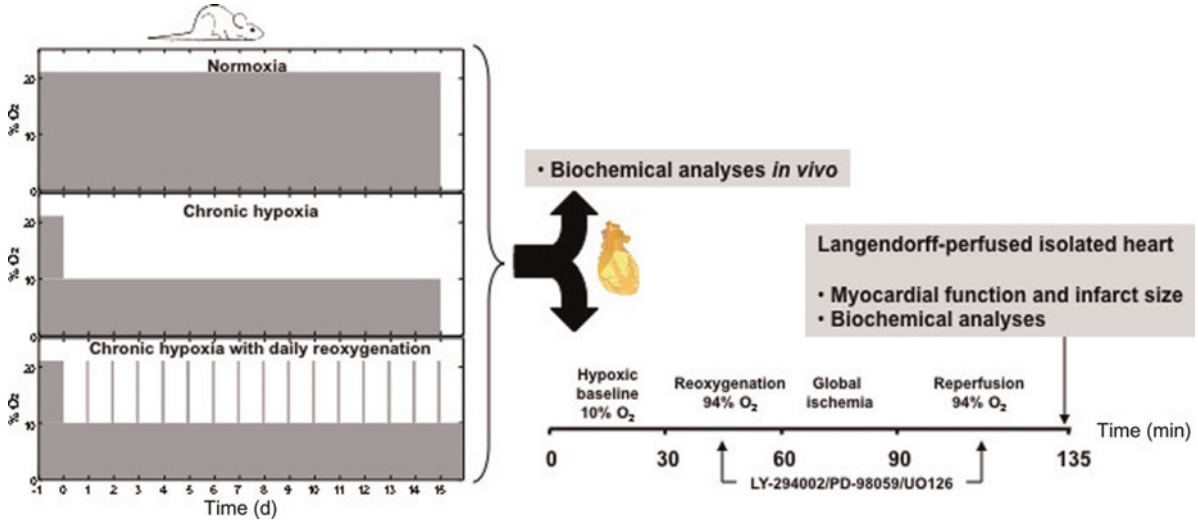

Figure 1 Scheme of the experimental protocol used in this study. Animals were exposed for 15 days to normoxia (control), chronic hypoxia or chronic hypoxia with daily reoxygenation. Then, hearts were either used for biochemical analyses or for Langendorff perfusion to assess performance and biochemistry after global ischemia and reperfusion. Some hearts from animals exposed to chronic hypoxia with reoxygenation were perfused in the presence of various inhibitors of the phosphorylation of phosphatidylinositol-3-kinase-protein kinase B or extracellular signal-regulated kinases 1/2 to assess the role of these proteins in cardioprotection (A color version of this figure is available in the online journal)

supernatant was transferred to precooled microcentrifuge tubes, frozen in liquid nitrogen and stored at −80°C. Total protein concentration was measured by bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). To perform sodium dodecyl sulfate-polyacrylamide gel electrophoresis Western blot, proteins (80 μg) were heated at 95°C for five minutes, electrophoresed on a 12% denaturating gel and electroblotted on to polyvinylidene fluoride membranes. Membranes were incubated with 5% non-fat dry milk in Tris-buffered saline-Tween buffer (1 h), followed by primary antibody (1/1000, 4°C, overnight) and horseradish peroxidase-conjugated secondary antibody (1/4000, room temperature, 1 h). Phosphorylated ERK1/2 (P-ERK1/2), Akt (P-Akt) and p38MAPK (P-p38) protein levels were determined with phosphospecific antibodies against P-ERK1/2 (Thr^{202}/Tyr^{204}), P-Akt (Ser^{473}) and P-p38MAPK (Thr^{180}/Tyr^{182}) (Cell Signaling Technologies, Allschwil, Switzerland). Loading of equal amount of proteins was verified by examining the intensity of the bands obtained with non-phosphospecific antibodies for ERK1/2, Akt and p38MAPK. Immunoblots were developed using a chemiluminescent system (LumiGlo reagent/peroxide; Cell Signaling Technologies). Band intensity was quantified by NIH AutoExtractor-1.51 software. The same extract from a normoxic heart was loaded on all blots for quantitative comparisons between blots.

HIF-1α immunohistochemistry

Biopsies from frozen myocardium were processed by embedding them in optimum cutting temperature compound (Leica Instruments, Nussloch, Germany) and serial 5-μm-thick sections were obtained using a cryomicrotome (Leica CM1510) and were placed on silanized glass slides. The sections were dried at room temperature for three minutes, fixed in 4% buffered formalin for 45 min at 4°C, rinsed two times for five minutes in PBS, postfixed with ethanol–acetic acid 2:1 (vol:vol) at −20°C for five minutes, rinsed twice for five minutes in PBS, boiled in 10 mmol/L citrate buffer at pH 6.0 for 10 min, washed once in distilled water and three times in PBS, and finally used for immunofluorescence. The sections were immersed in 10% normal goat serum for one hour with gentle agitation, incubated overnight at 4°C with the primary mouse anti-HIF-1α monoclonal antibody (Chemicon International, Temecula, CA, USA; diluted 1:400 in 1.5% normal goat serum), washed in PBS and incubated at room temperature for 45 min with the secondary goat antimouse IgG fluorescein-conjugated antibody (Santa Cruz Biotechnology, Nunningen, Switzerland; diluted 1:200 in 1.5% normal goat serum). A negative control was prepared for each section by substituting the primary antibody with 1.5% normal goat serum. The slides were examined at ×40 magnification in an inverted fluorescence microscope (Axiovert 25 CFL; Carl Zeiss, Göttingen, Germany), equipped with a filter for the detection of fluorescein (filter set 09, excitation band pass 450–490 nm, emission low pass 515 nm). The images were acquired by a Digital Camera System for Microscopy (DS-2Mv; Nikon Corporation, Tokyo, Japan), and stored in a personal computer. For the quantification of the signals, the images were analyzed by IPlab Software (Scanalytics Inc, Billerica, MA, USA) and split into RGB channels. The green channel was used to calculate the color intensity as the sum of the pixel intensity values. At least five random fields per each section were selected, the total color intensity was measured and subtracted of the signal detected using the negative controls. The color intensity in the image is expressed as the sum of pixel intensity $\times 10^6/0.037\ mm^2$.

Langendorff heart perfusion

The freshly removed heart was immersed in a beaker containing de-aerated isotonic saline at 25°C; the beaker was taken out of the chamber, the aorta immediately cannulated on the perfusion system and the heart perfused at 37°C with the hypoxic medium. The time interval between heart excision and mounting on the perfusion system was <90 s. The fixed-flow (15 mL/min) Langendorff perfusion apparatus is described elsewhere.[1] The gas (Carbagas, Lausanne, Switzerland) flowing through the membrane microoxygenator (Dideco, Mirandola, Italy) contained O_2, CO_2 and N_2 in the proportion 10/6/84 or 94/6/0 for the hypoxic perfusion, or reoxygenation and reperfusion, respectively. The temperature of the medium (Krebs–Henseleit buffer with 2.0 mmol/L free Ca^{2+} and 11 mmol/L glucose, pH 7.40 ± 0.01) and heart chamber was 37°C. A latex balloon in the left ventricle was connected to a pressure transducer (MPC-500; Millar Instruments Inc, Houston, TX, USA) to monitor performance. An additional transducer above the aortic cannula monitored the coronary perfusion pressure (CPP).

Experimental protocol

Hearts were stabilized for 30 min at $PO_2 = 67$ mmHg, corresponding to 10% O_2. During this period, the balloon volume was set to diastolic pressure (DP) = 10 mmHg and kept constant afterwards. At the end of the hypoxic stabilization, hearts were subjected to 30 min reoxygenation (CHR) or oxygenation (CH) at $PO_2 = 670$ mmHg, followed by 30 min global ischemia (switching off the pump) and 45 min reperfusion under the same conditions of reoxygenation. Myocardial performance was monitored continuously, but for clarity we report data every five minutes. We measured DP, heart rate (HR), peak systolic pressure (PSP), left ventricle developed pressure (LVDP, or PSP-DP) and the developed pressure × heart rate product (LVDPxHR).

To test the involvement of Akt and ERK1/2, part of CHR hearts were perfused with 15 μmol/L LY-294002 ($n = 10$), 10 μmol/L UO-126 ($n = 10$) or 10 μmol/L PD-98059 ($n = 9$). LY-294002 and PD-98059 are considered selective inhibitors of the phosphorylation of Akt and ERK1/2, respectively,[8] with UO-126 showing greater specificity for ERK1/2 than PD-98059 because it acts directly on MAP kinase kinase and ERK1/2.[9] The infusion of the substances started 15 min before global ischemia and ended 20 min after the beginning of reperfusion. LY-294002, PD-98059 (Alexis Corporation, Lausen, Switzerland) and UO-126 (LC Laboratories, Boston, MA, USA) were initially dissolved in a small volume of dimethyl sulfoxide (DMSO) and

diluted in the medium immediately before administration (final DMSO concentration in the perfusion medium $<0.01\%$ (v/v)).

At the end of the perfusion, hearts were either used to determine the level of ERK1/2, P-ERK1/2, Akt, P-Akt, p38MAPK and P-p38MAPK as described above ($n = 27$), or the infarct size ($n = 38$). For the latter measurement, the ventricles were cut into one-mm transverse sections from apex to base (5–6 slices/heart). Once thawed, the slices were incubated at 37°C with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) for 20 min and fixed in 10% formalin for four days to enhance the contrast between stained (viable) and unstained (damaged) areas. The infarct size was determined from imaging the slices (NIH Image AutoExtractor 1.51) and expressed as percentage of ventricular size.

Statistics

Data are expressed as mean ± standard error of mean. Significance level was $P = 0.05$ (two tailed). To detect differences among the groups, we performed one-way analysis of variance. If this test resulted significant, the differences between selected pairs of data were tested using the Bonferroni procedure. When comparing two groups, this test reduces to the Student's *t*-test for unpaired data.

Results

In agreement with previous studies,[2] whereas CH rats had a negative weight balance, CHR rats gained weight during the 15-day observation period, although less than N rats (Table 1). The heart/body weight ratio was higher for CH and CHR rats than for N rats, which indicates greater degree of myocardial hypertrophy independently of the mode of hypoxia. Both hypoxia conditions led to the same degree of polycythemia. Plasma nitrates + nitrites, a marker of NO production, was higher in CHR than CH rats.

Daily reoxygenation ameliorates post-I/R performance and tolerance to injury

In isolated hearts perfused by the Langendorff method at constant volume of the intraventricular balloon and constant flow, the increase in DP and CPP, with depression in LVDPxHR marks the onset of diastolic stiffness, vasoconstriction and the deterioration of myocardial performance, respectively. After I/R, when performance was stable, DP was less and LVDPxHR was higher in CHR than CH hearts, whereas CPP was essentially maintained in the three groups (Figure 2). The right panels in Figure 2 show the variation of the considered parameters at the end of the reperfusion with respect to the values monitored at the end of either the baseline or the reoxygenation. Thus, daily reoxygenation enabled hearts to perform at a better level after I/R. However, whereas the protection afforded to DP was effective for the full duration of the protocol, the protection of LVDPxHR was almost completely exhausted at the end of the reoxygenation. No protection was observed for CPP/heart weight.

At the end of the reperfusion, hearts were harvested and the size of the infarct measured to obtain an index of myocardial injury. The size of the infarct was less in CHR than CH hearts ($25 \pm 2\%$ versus $40 \pm 3\%$, $P = 0.003$). Therefore, daily reoxygenation not only enabled better myocardial performance, but also reduced markedly the injury led by I/R.

Daily reoxygenation increases the *in vivo* phosphorylation rates of Akt and ERK1/2 and blunts the hypoxia-induced increase in HIF-1α

Next, we determined whether CH and CHR influence differentially the expression level and the phosphorylation rate of Akt and ERK1/2 in the *in vivo* heart. To this purpose, CH rats were sacrificed in the compensation chamber under 10% O_2. Thus, CH data refer to hypoxic tissue that was never subjected to the reoxygenation. CHR rats were sacrificed in the same way 24 h after the 15th daily reoxygenation event. Thus, CHR data also refer to hypoxic tissue and can thus be compared with CH, but were obtained in rats that have been repeatedly reoxygenated. N rats were sacrificed in normoxia.

Total expression of Akt and ERK1/2 remained unaffected by either CH or CHR (Figure 3). By contrast, both P-Akt and P-ERK1/2 were higher in CHR than CH hearts. Therefore, the P-Akt/Akt ratio changed from 0.9 ± 0.1 in N hearts to 0.6 ± 0.1 and to 1.3 ± 0.1 ($P = 0.02$) in CH and CHR hearts, respectively. The P-ERK1/2/ERK1/2 was 0.6 ± 0.1 in N hearts, and 0.6 ± 0.1 and 0.9 ± 0.1 ($P = 0.0005$) in CH and CHR hearts, respectively. Thus, daily reoxygenation increased the *in vivo* phosphorylation rate of both Akt and ERK1/2. By contrast, the phosphorylation of p38MAPK was influenced by CH but not by CHR. As expected,[4] CH induces HIF-1α overexpression in the myocardium, and

Table 1 Weight and blood values in normoxia, chronic hypoxia and chronic hypoxic with daily reoxygenation

	Normoxia	Chronic hypoxia	Chronic hypoxia with daily reoxygenation
Body weight gain (g)	89 ± 4	$-2 \pm 6^{*}$	$39 \pm 6^{*,**}$
Heart weight (g)	1.18 ± 0.02	$1.30 \pm 0.04^{*}$	$1.54 \pm 0.05^{*,**}$
Heart/body weight (mg/g)	3.6 ± 0.1	$5.5 \pm 0.2^{*}$	$5.3 \pm 0.1^{*}$
Hematocrit (%)	48 ± 2	$70 \pm 3^{*}$	$68 \pm 2^{*}$
Hemoglobin (g/L)	145 ± 3	$200 \pm 4^{*}$	$204 \pm 4^{*}$
Red blood cell count (RBC/μL/1000)	7.7 ± 0.3	$9.8 \pm 0.2^{*}$	$9.3 \pm 0.3^{*}$
Nitrates + nitrites (μmol/L)	4.2 ± 0.3	$8.7 \pm 0.9^{*}$	$13.3 \pm 1.6^{*,**}$

Data are mean ± standard error of mean

$^{*}P < 0.05$ versus normoxia

$^{**}P < 0.05$ versus chronic hypoxia (Fischer's post-test)

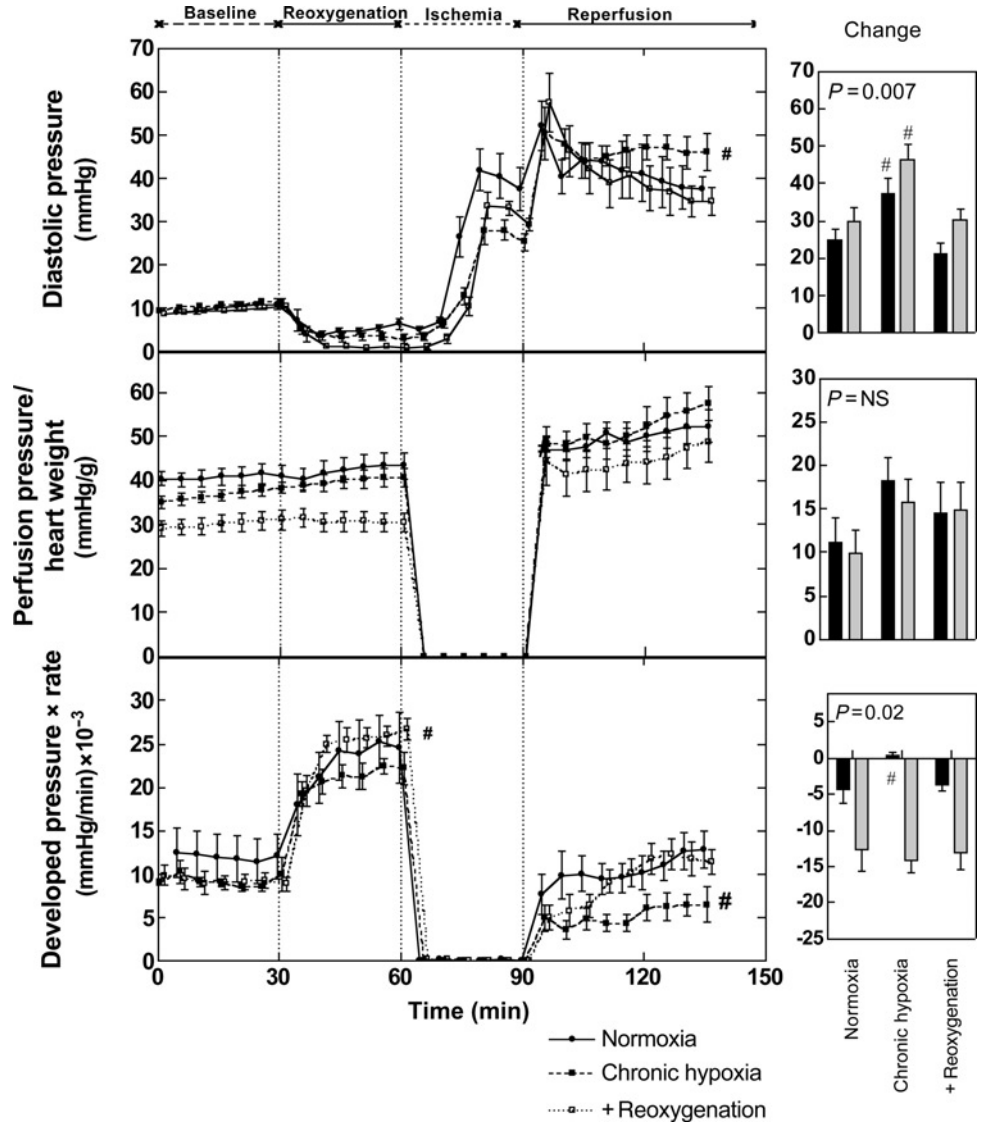

Figure 2 Daily reoxygenation improves myocardial tolerance to ischemia/reperfusion. The left panels show the time courses for diastolic pressure (upper panel), coronary perfusion pressure as its ratio with heart weight (middle panel) and the product (left ventricle developed pressure) × (heart rate) (lower panel) during the ischemia/reperfusion protocol. Data are expressed as mean ± standard error of mean; $n = 7$/group. For the sake of clarity, the statistical analysis refers only to the values measured at the end of the various phases. $^{\#}P < 0.05$ versus chronic hypoxia with reoxygenation, Student's *t*-test. The right panels show the changes of the respective parameters calculated either as end reperfusion minus baseline (filled bars) or endreperfusion minus reoxygenation (gray bars). The inset represents the one-way analysis of variance test. $^{\#}P < 0.05$ versus chronic hypoxia with reoxygenation, Bonferroni post-test

Figure 3 shows representative immunohistochemistry images taken from hearts in the three groups under study. However, in CHR hearts HIF-1α overexpression was markedly blunted by returning near normoxic values.

LY-29002, PD-98059 and UO-126 inhibit differentially the phosphorylation rate of Akt and ERK1/2

To examine the specific roles of P-Akt and P-ERK1/2 on cardioprotection, we tested whether inhibiting the phosphorylation of these proteins during I/R in isolated hearts blunts the protective effect of CHR. We first examined the selectivity of LY-29002, UO-126 and PD-98059 when given to perfused hearts starting 15 min before global ischemia and ending 20 min after the beginning of reperfusion. To this purpose, we determined the phosphorylation rate of Akt and ERK1/2 in biopsies taken from perfused hearts at the end of the reperfusion (Figure 4). Whereas the administration of 10 μmol/L PD-98059 decreased the phosphorylation of both Akt and ERK1/2 compared with untreated CHR, the administration of 15 μmol/L LY-294002 decreased the phosphorylation of Akt without effects on ERK1/2. By contrast, administration of UO-126 decreased the phosphorylation of ERK1/2 without effects on Akt. All the tested inhibitors did not have any effect on the phosphorylation of p38MAPK.

LY-29002, PD-98059 and UO-126 antagonize the protective effect of daily reoxygenation

CHR hearts were exposed to I/R in the presence of these inhibitors at the reported times and concentrations, while

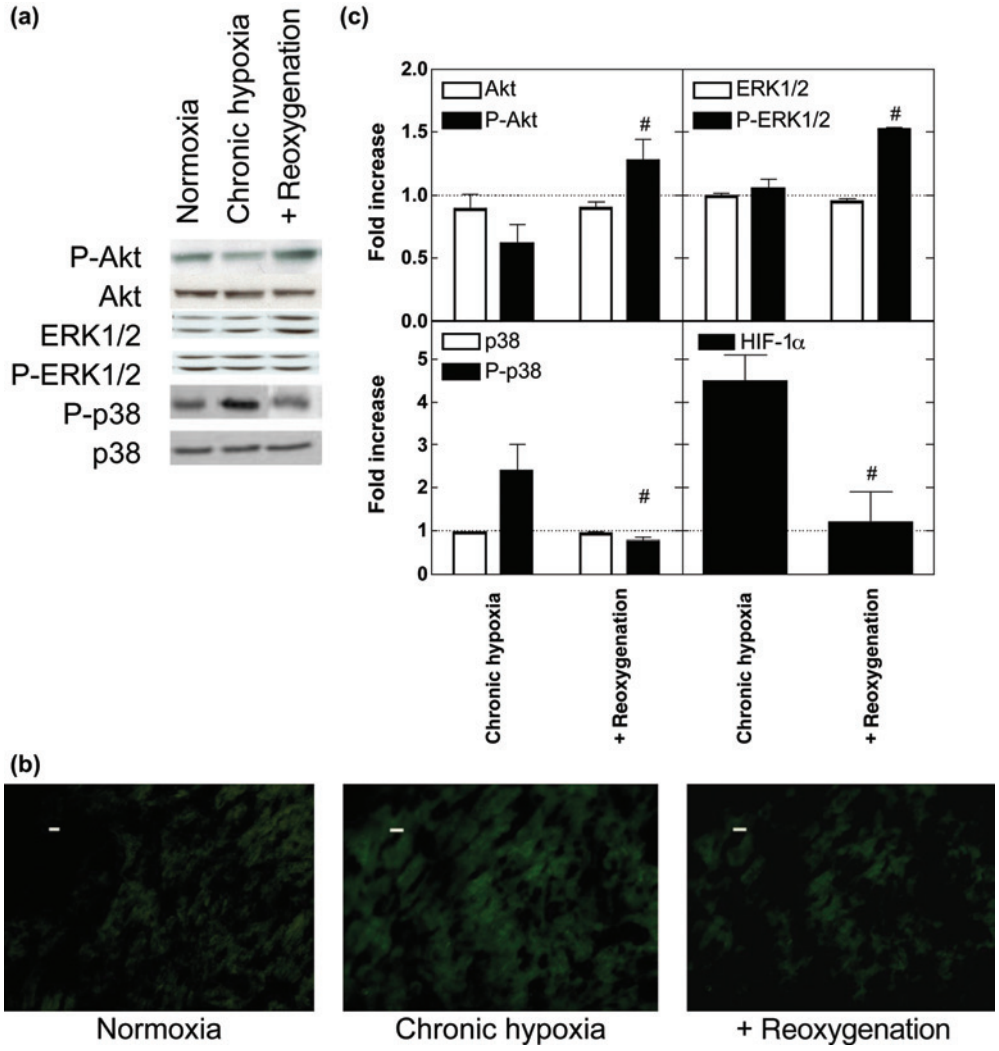

Figure 3 Daily reoxygenation increases the phosphorylated forms of phosphatidylinositol-3-kinase-protein kinase B (Akt) and extracellular signal-regulated kinases (ERK1/2) *in vivo*, and blunts hypoxia-inducible factor-1α (HIF-1α) and the phosphorylation of p38 mitogen-activated protein kinase (MAPK). (a) Representative Western blots for the phosphorylated and total isoforms of Akt, ERK1/2 and p38MAPK from rats exposed to normoxia, chronic hypoxia or chronic hypoxia with daily reoxygenation for 15 days. The intensity of the blots from five hearts for each group was measured by densitometry, averaged and the phosphorylated/total protein ratio expressed as fold increase over normoxia, reported in panel c. (b) Representative HIF-1α immunofluorescence staining images taken from left ventricle biopsies in hearts from the three groups. The white bar represents 20 μm. (c) The quantification of the immunofluorescence staining, expressed as intensity of green pixels measured in all animals (mean $\pm$ standard error) (same statistics as above). Data in panel c are expressed as mean $\pm$ standard error of mean; $n = 5$/group. The inset reports the analysis of variance $P < 0.05$ for all. $^{\#}P < 0.05$ versus chronic hypoxia, Bonferroni post-test

monitoring performance and tolerance to I/R. Figure 5 shows that LY-29002, PD-98059 and UO-126 increased DP from 31.7 ± 3.1 mmHg in untreated CHR to 67.5 ± 9.4, 50.9 ± 2.3 and 48.9 ± 6.4 mmHg, respectively. Thus, all inhibitors markedly reversed the protective effect of CHR on the development of diastolic stiffness. However, the increase in DP was less in the presence of both PD-98059 and UO-126. The inhibitors also reduced LVDPxHR from 12.8 ± 1.6 mmHgx10^3/min in untreated CHR to 8.0 ± 0.8, 7.9 ± 0.7 and 8.3 ± 0.4 mmHgx10^3/min versus CHR + LY-29002, CHR + PD-98059 and CHR + UO-126, respectively. Thus all inhibitors reversed the protection of myocardial performance afforded by CHR by the same extent. CPP markedly elevated, a sign of greater vascular damage, in the hearts treated with LY-29002. In hearts treated with PD-98059, there was no significant exacerbation of the perfusion pressure. Hearts treated with UO-126 displayed an intermediate pattern between the two. Finally, the infarct size increased from $25 \pm 2\%$ in untreated CHR to $41 \pm 4\%$, $40 \pm 4\%$ and $39 \pm 5\%$, respectively, indicative of loss of the protection given by CHR.

Discussion

In vivo daily reoxygenation during CH enhances myocardial Akt and ERK1/2 signaling. This biochemical response was accompanied by a complex phenotype consisting in improved resistance to stress, better myocardial performance and lower infarct size after I/R. In fact, essentially all the parameters observed to evaluate cardioprotection were impaired in CH, but returned to near normoxic levels in CHR hearts. Selective inhibition of the phosphorylation of Akt and ERK1/2, however, abolished the beneficial effects of daily reoxygenation. Therefore, Akt and ERK1/2 have

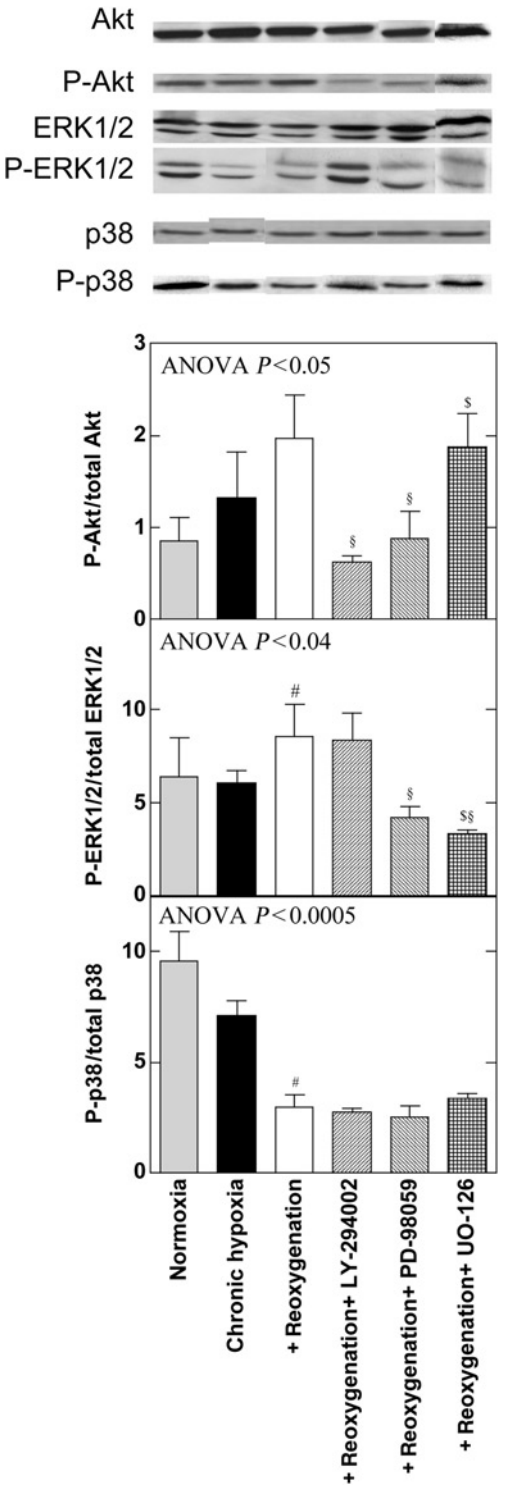

Figure 4 Inhibition with LY-294002, PD-98059 or UO-126 selectively decreases the phosphorylation rate of phosphatidylinositol-3-kinase-protein kinase B (Akt) and extracellular signal-regulated kinases (ERK1/2) at the end of the reperfusion. Western blots for total and phosphorylated Akt and ERK1/2 were performed in the left ventricle at the end of the ischemia/reperfusion protocol (representative blots shown in the upper panel); the intensity of the blots was measured by densitometry, averaged and reported in the bottom panel as phosphorylated/total protein ratio. Data are expressed as mean ± standard error of mean; $n = 4–5$/group. The inset reports the analysis of variance *P*. $^{\#}P < 0.05$ versus chronic hypoxia; $^{\S}P < 0.05$ versus chronic hypoxia with daily reoxygenation; and $^{\$}P < 0.05$ versus chronic hypoxia with daily reoxygenation treated with LY-294002, Bonferroni post-test

an important role in determining the cardioprotection elicited by daily reoxygenation during CH *in vivo*.

We previously demonstrated the cardioprotective effect of daily one-hour aeration of the animals during CH, which is independent of blood gases, hemoglobin concentration.[1] Furthermore, we showed that ventricular hypertrophy, although higher in CH with respect to normoxia, was unaffected by the reoxygenation.[5] Thus, the main factor that may account for the differential effects of CH and CHR appears to be related to the ox–redox balance, because the plasma level of α-tocopherol supports increased oxidative stress with animal aeration.[1] The association of CHR with greater oxidative stress than CH is confirmed, in part, by the observation that intermittent hypoxic training augments blood lipid hydroperoxides and malondialdehyde, and that this increase is attenuated in humans exercising regularly under hypoxic conditions.[10] In rat plasma, α-tocopherol concentration is less in CHR than CH, as a consequence of the greater oxidative stress induced by daily reoxygenation. The level of α-tocopheryl phosphate is probably linked to cardioprotection via Akt activation.[11] Here, we show that the phosphorylation of Akt and ERK1/2 is functional to activate this link.

The characteristics of the Langendorff-perfused heart model were discussed elsewhere.[12] Although limited by low mitochondrial oxygen tension as compared with *in vivo* hearts,[13] as well as by premature phosphorylation of ERK1/2 (but not Akt) during heart handling operations,[14] the *ex vivo* Langendorff model allows identification of the specific roles of signaling paths in myocardial tissue without potentially confounding factors correlated with the circulation. In the selected configuration, total coronary flow has been maintained constant throughout. As CHR hearts (and to a minor extent CH hearts) were hypertrophic with respect to N, this necessarily implies lower specific coronary flow in CHR, which may affect I/R tolerance *per se*. However, despite the possible occurrence of this confounding variable, the coronary pressure/heart weight did not display significant differences in the various groups. However, we cannot rule out the possibility that hypertrophic CHR hearts have been perfused throughout at a lower O_2 supply/g tissue ratio than N and CH. This might have affected the final ischemia tolerance. However, as there is no effect of I/R on perfusion pressure/heart weight, it is likely that the effect of varying specific coronary flows would not be too critical with respect to ischemia tolerance.

The employed protocol (hypoxic baseline, reoxygenation, global I/R) was selected for the following reasons. First, for consistency with the previous studies by our group that reported greater tolerance in CHR hearts, but without clarifying the underlying mechanisms.[2,12] Second, this protocol enables separating the effects due to (re)oxygenation from those due to I/R. Indeed, the protection afforded by the (re)oxygenation to the diastolic function appeared effective for the whole duration of the Langendorff perfusion. By contrast, the protection afforded to myocardial performance (or LVDPxHR) was effective only until the end of the reoxygenation phase. It remains to be established if this was a consequence of the reoxygenation injury or of excessively elapsed time. In any case, this implies that the molecular

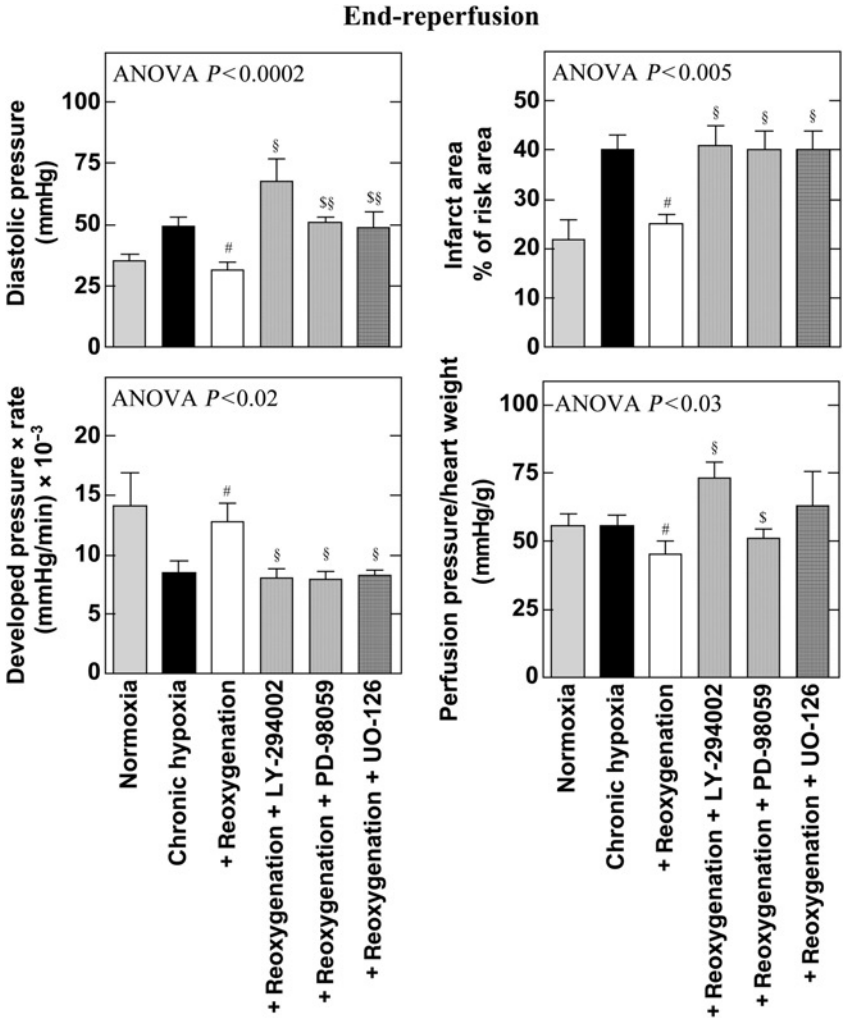

Figure 5 Daily reoxygenation improves myocardial performance and reduces the infarct size after ischemia–reperfusion. The panels show (clockwise from top left) the diastolic pressure, the infarct size at the end of the reperfusion, the ratio perfusion pressure/heart weight and the product of left ventricle developed pressure × heart rate, with/without inhibitors. Data are expressed as mean ± standard error of mean; $n = 5–7$/group. The inset reports analysis of variance *P*. $^{\#}P < 0.05$ versus chronic hypoxia; $^{\S}P < 0.05$ versus chronic hypoxia with daily reoxygenation; and $^{\$}P < 0.05$ versus chronic hypoxia with daily reoxygenation treated with LY-294002, Bonferroni post-test

mechanisms underlying protection may have different phenotypes depending on which myocardial function is being examined. Such analysis would not have been possible for CH hearts, which were continuously hypoxic for 15 days, if the hypoxic baseline step were avoided. In these hearts, exposure to hyperoxic perfusion just after removal from the animals would have caused abrupt oxygenation with severe premature injury that would zero such differences. Third, despite the fact that it is likely that 20-min hypoxic perfusion might stimulate protective pathways, the persistence of the protective effects throughout Langendorff perfusion with respect to DP suggests that hypoxic preconditioning-like signaling might not be a critical factor during the *in vitro* protocol.

Hypoxia *in vivo* is known to up-regulate HIF-1α, which transactivates an array of genes involved in many cell pathways, including anaerobic metabolism, angiogenesis and apoptosis.[15] Remarkably, we found that HIF-1α protein overexpression was quenched in CHR hearts, in agreement with previous *in vivo* data from our group showing fast normalization of HIF-1α signal upon reoxygenation,[16] as well as recent work *in vitro*.[17] This suggests that HIF-1α signaling is unnecessary for Akt and ERK1/2 phosphorylation *in vivo*. As neither Akt nor ERK1/2 are under the control of HIF-1α, the lack of effect of CH and CHR on the expression level of Akt and ERK1/2 total protein is not surprising. The phosphorylation of both Akt and ERK1/2 was increased in CHR, much similar to as reported in human vascular endothelial cells exposed to cyclic hypoxia.[17] By contrast, the phosphorylation of Akt was decreased in CH while ERK1/2 did not change. Thus, it is likely that the oxidative stress associated with CHR increased the phosphorylation state of those proteins. Existing literature is controversial. On one hand, rats exposed to long-term intermittent hypoxia (12% O_2, 8 h/day for 4 and 8 weeks) exhibited decreased Akt phosphorylation and increased apoptosis, with possible involvement of both mitochondrial-dependent and Fas death receptor-dependent apoptotic paths.[18] Also, in cultured cardiomyocytes subjected to hypoxia and reoxygenation, induction of gene 33 mRNA and gene 33 protein reduces Akt and ERK1/2 signaling.[19] By contrast, hypoxia/reoxygenation in renal epithelial cells

activates both ERK1/2 and Akt survival signaling paths through reactive oxygen species (ROS) signaling and the Ras/Raf cascade.[20] Essentially similar features were found in human vascular endothelial cells exposed to cyclic hypoxia.[17] Another intriguing report suggests that hypoxia might activate ERK1/2 through stimulation of erythropoietin receptors and two synergic signaling paths, one of which bypasses Akt, whereas the other involves downstream dependency of ERK1/2 on Akt.[21] At present, it is difficult to understand the reason(s) for this discrepancy, but it has been demonstrated that, in endothelial cells subjected to oxidative stress, the Akt and ERK1/2 pathways serve as signaling mediators to alleviate H_2O_2 cytotoxic effects and function in parallel to mediate prosurvival signaling pathways.[22] Increased phosphorylation of Akt and ERK1/2 in CHR animals is associated with improved tolerance to I/R, in agreement with observations on the role of Akt in mitochondrial survival pathways triggered by hypoxic preconditioning.[23] ROS may act as trigger for Akt and ERK phosphorylation.[17,20,24,25] Reactivation of Akt has also been recognized as a critical determinant of survival in posthypoxic cardiomyocytes in culture.[26] Finally, ERK1/2 and Akt are required for erythropoietin-induced neuroprotection.[27]

The protective effect of CHR was blunted by blockade of either Akt with LY-294002 or ERK1/2 with PD98059. This supports a direct role for Akt and ERK1/2 in mediating cardioprotection. The observed beneficial effects of Akt activation on cardioprotection agree with a study on the involvement of this pathway in the cardioprotective effect of chronic intermittent hypobaric hypoxia (8 h/day, 25–30 exposures).[28] That study showed that Akt inhibition completely abolished anti-infarct effect of IP and exacerbated injury in rats adapted to intermittent hypobaric hypoxia, suggesting that activation of this cascade might represent a potential common route in eliciting preconditioning, for example, by activating the endothelial isoform of NO synthase[29] and the NO/cGMP pathway, as it was found in the brain.[30] In this study, the administration of PD-98059, a putative antagonist of ERK1/2, blocked CHR-induced phosphorylation of both ERK1/2 and Akt, whereas LY-294002, an antagonist of Akt, blocked phosphorylation of Akt only. This apparently contradictory trend can be explained on the basis of the downstream dependency of Akt on ERK1/2 that was observed in a model of IP in mice knocked out for mitogen-activated protein kinase activated protein kinase 2.[31] Furthermore, it was shown that LY-294002 might inhibit K^+_{ATP} channels, thereby providing an extra way to protect hearts.[32] The same authors nevertheless warn that the use of LY294002 is limited by its nonspecific effects, and this limitation is therefore valid in the present study too. However, it is not clarified how exactly K^+_{ATP} channels blockade exert their protective effect, because it was also shown that these channels are activated upstream of ERK1/2 in a model of preconditioning activated by hydrogen sulfide.[33] Thus, translating ERK1/2 and/or Akt phosphorylation into a cardioprotective effect needs a word of caution, but our data clearly show that the ventricular function and the injury after I/R is challenged by both inhibitors by the same extent, whereas LY-294002 appears more challenging than PD-98059 as far as the diastolic (DP) and vascular (CPP) components of the reperfusion injury are considered, suggesting that the protective mechanisms elicited by inhibiting Akt and ERK1/2 are different.

p38MAPK activity down-regulation after CHR with respect to CH *in vivo* (Figure 3) is consistent with previous observations under similar conditions.[3] Because such down-regulation persisted in hearts that were removed from the animal, Langendorff-perfused and exposed to I/R (Figure 4), it is tempting to speculate that this down-regulation causes protection in CHR hearts, because p38MAPK during ischemia has been shown to reduce infarct by mediating IP.[34] However, the employed inhibitors did not affect p38MAPK activity, yet they blunted the protection afforded by CHR, ruling out p38MAPK down-regulation in the cardioprotection afforded by CHR.

Timing of kinases inhibition is critical to assess their roles as trigger or mediator of protection. Previous studies, summarized in reference,[34] distinguished these two roles of the various kinases by modifying the time of application of the respective inhibitors: during the trigger phase (in our case, in *in vivo* animals) or during the mediator phase (in our case, in the perfused hearts before I/R). As in the present study, the infusion of the inhibitors started 15 min before ischemia and ended 20 min after the beginning of reperfusion; this resembles the paradigm whereby ERK1/2 and Akt act as mediators rather than triggers of protection, which fits with the current ideas on the signal cascade of IP.

Conclusions

While CH depresses myocardial tolerance to I/R, daily reoxygenation alleviates the derangement induced by hypoxia. Such mechanism resembles that elicited during the so-called 'second window of protection' as it recruits a variety of cell mechanisms synergistic in mediating, and not triggering, the protection. The pathways elicited by Akt and, presumably to a lesser extent, by ERK1/2 appear to be critically involved in the preconditioning effect induced by daily reoxygenation during CH. Possibly, some events associated with the reoxygenation, for example the oxidative stress or ROS, increase the phosphorylation of Akt and ERK1/2, thereby creating the basis for the cardioprotection induced by daily reoxygenation during CH.

ACKNOWLEDGEMENTS

This study has been supported, in part, by Foundation Andreas P Naef de la Chirurgie Thoracique, Société Académique Vaudoise, Lausanne, Switzerland, and in part by Swiss National Science Foundation (FN 310000–110058/1) and Italian MIUR (PRIN Project 2007).

REFERENCES

1 Milano G, Corno A, Lippa S, von Segesser L, Samaja M. Chronic and intermittent hypoxia induce different degrees of myocardial tolerance to hypoxia-induced dysfunction. *Exp Biol Med* 2002;**227**:389–97

2 Milano G, Bianciardi P, Corno AF, Raddatz E, Morel S, von Segesser LK, Samaja M. Myocardial impairment in chronic hypoxia is abolished by short aeration episodes: involvement of K^+ATP channels. *Exp Biol Med (Maywood)* 2004;**229**:1196–205

3 Morel S, Milano G, Ludunge KM, Corno AF, Samaja M, Fleury S, Bonny C, Kappenberger L, von Segesser LK, Vassalli G. Brief reoxygenation episodes during chronic hypoxia enhance posthypoxic recovery of LV function: role of mitogen-activated protein kinase signaling pathways. *Basic Res Cardiol* 2006;**101**:336–45

4 Bianciardi P, Fantacci M, Caretti A, Ronchi R, Milano G, Morel S, von Segesser L, Corno A, Samaja M. Chronic *in vivo* hypoxia in various organs: hypoxia-inducible factor-1alpha and apoptosis. *Biochem Biophys Res Commun* 2006;**342**:875–80

5 Corno AF, Milano G, Morel S, Tozzi P, Genton CY, Samaja M, von Segesser LK. Hypoxia: unique myocardial morphology? *J Thorac Cardiovasc Surg* 2004;**127**:1301–8

6 Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia–reperfusion injury: targeting the reperfusion injury salvage kinase (RISK)-pathway. *Cardiovasc Res* 2004;**61**:448–60

7 Verdon C, Burton B, Prior R. Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by $NADP^+$ when the Griess reaction is used to assay for nitrate. *Anal Biochem* 1995;**224**:502–8

8 Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, Przyklenk K. Postconditioning via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK1/2. *Am J Physiol Heart Circ Physiol* 2005;**289**:H1618–26

9 Duncia JV, Santella JB III, Higley CA, Pitts WJ, Wityak J, Frietze WE, Rankin FW, Sun JH, Earl RA, Tabaka AC, Teleha CA, Blom KF, Favata MF, Manos EJ, Daulerio AJ, Stradley DA, Horiuchi K, Copeland RA, Scherle PA, Trzaskos JM, Magolda RL, Trainor GL, Wexler RR, Hobbs FW, Olson RE. MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. *Bioorg Med Chem Lett* 1998;**8**:2839–44

10 Bailey DM, Davies B, Young IS. Intermittent hypoxic training: implications for lipid peroxidation induced by acute normoxic exercise in active men. *Clin Sci (Lond)* 2001;**101**:465–75

11 Mukherjee S, Lekli I, Das M, Azzi A, Das DK. Cardioprotection with alpha-tocopheryl phosphate: Amelioration of myocardial ischemia reperfusion injury is linked with its ability to generate a survival signal through Akt activation. *Biochim Biophys Acta* 2008;**1782**:498–503

12 Milano G, Corno AF, Lippa S, Von Segesser LK, Samaja M. Chronic and intermittent hypoxia induce different degrees of myocardial tolerance to hypoxia-induced dysfunction. *Exp Biol Med (Maywood)* 2002;**227**:389–97

13 Mik EG, Ince C, Eerbeek O, Heinen A, Stap J, Hooibrink B, Schumacher CA, Balestra GM, Johannes T, Beek JF, Nieuwenhuis AF, van Horssen P, Spaan JA, Zuurbier CJ. Mitochondrial oxygen tension within the heart. *J Mol Cell Cardiol* 2009;**46**:943–51

14 Stenslokken KO, Rutkovskiy A, Kaljusto ML, Hafstad AD, Larsen TS, Vaage J. Inadvertent phosphorylation of survival kinases in isolated perfused hearts: a word of caution. *Basic Res Cardiol* 2009;**104**:412–23

15 Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG, Semenza GL. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood* 2005;**105**:659–69

16 Caretti A, Morel S, Milano G, Fantacci M, Bianciardi P, Ronchi R, Vassalli G, von Segesser LK, Samaja M. Heart HIF-1alpha and MAP kinases during hypoxia: are they associated *in vivo*? *Exp Biol Med (Maywood)* 2007;**232**:887–94

17 Martinive P, Defresne F, Quaghebeur E, Daneau G, Crokart N, Gregoire V, Gallez B, Dessy C, Feron O. Impact of cyclic hypoxia on HIF-1alpha regulation in endothelial cells – new insights for anti-tumor treatments. *FEBS J* 2009;**276**:509–18

18 Lee SD, Kuo WW, Lin JA, Chu YF, Wang CK, Yeh YL, Wang SG, Liu JY, Chang MH, Huang CY. Effects of long-term intermittent hypoxia on mitochondrial and Fas death receptor dependent apoptotic pathways in rat hearts. *Int J Cardiol* 2006;**116**:348–56

19 Xu D, Patten RD, Force T, Kyriakis JM. Gene 33/RALT is induced by hypoxia in cardiomyocytes, where it promotes cell death by suppressing phosphatidylinositol 3-kinase and extracellular signal-regulated kinase survival signaling. *Mol Cell Biol* 2006;**26**:5043–54

20 Kwon DS, Kwon CH, Kim JH, Woo JS, Jung JS, Kim YK. Signal transduction of MEK/ERK and PI3K/Akt activation by hypoxia/reoxygenation in renal epithelial cells. *Eur J Cell Biol* 2006;**85**:1189–99

21 Miki T, Miura T, Tanno M, Nishihara M, Naitoh K, Sato T, Takahashi A, Shimamoto K. Impairment of cardioprotective PI3K-Akt signaling by post-infarct ventricular remodeling is compensated by an ERK-mediated pathway. *Basic Res Cardiol* 2007;**102**:163–70

22 Yang B, Oo TN, Rizzo V. Lipid rafts mediate H_2O_2 prosurvival effects in cultured endothelial cells. *FASEB J* 2006;**20**:1501–3

23 Uchiyama T, Engelman RM, Maulik N, Das DK. Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. *Circulation* 2004;**109**:3042–9

24 Budisavljevic MN, Hodge L, Barber K, Fulmer JR, Durazo-Arvizu RA, Self SE, Kuhlmann M, Raymond JR, Greene EL. Oxidative stress in the pathogenesis of experimental mesangial proliferative glomerulonephritis. *Am J Physiol Renal Physiol* 2003;**285**:F1138–48

25 Wang X, McCullough KD, Franke TF, Holbrook NJ. Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. *J Biol Chem* 2000;**275**:14624–31

26 Shao Z, Bhattacharya K, Hsich E, Park L, Walters B, Germann U, Wang YM, Kyriakis J, Mohanlal R, Kuida K, Namchuk M, Salituro F, Yao YM, Hou WM, Chen X, Aronovitz M, Tsichlis PN, Bhattacharya S, Force T, Kilter H. c-Jun N-terminal kinases mediate reactivation of Akt and cardiomyocyte survival after hypoxic injury *in vitro* and *in vivo*. *Circ Res* 2006;**98**:111–8

27 Kilic E, Kilic U, Soliz J, Bassetti CL, Gassmann M, Hermann DM. Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways. *FASEB J* 2005;**19**:2026–8

28 Ravingerova T, Matejikova J, Neckar J, Andelova E, Kolar F. Differential role of PI3K/Akt pathway in the infarct size limitation and antiarrhythmic protection in the rat heart. *Mol Cell Biochem* 2007;**297**:111–20

29 Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;**399**:601–5

30 Caretti A, Bianciardi P, Ronchi R, Fantacci M, Guazzi M, Samaja M. Phosphodiesterase-5 inhibition abolishes neuron apoptosis induced by chronic hypoxia independently of hypoxia-inducible factor-1alpha signaling. *Exp Biol Med (Maywood)* 2008;**233**:1222–30

31 Nagy N, Shiroto K, Malik G, Huang CK, Gaestel M, Abdellatif M, Tosaki A, Maulik N, Das DK. Ischemic preconditioning involves dual cardio-protective axes with p38MAPK as upstream target. *J Mol Cell Cardiol* 2007;**42**:981–90

32 Oudit GY, Sun H, Kerfant BG, Crackower MA, Penninger JM, Backx PH. The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *J Mol Cell Cardiol* 2004;**37**:449–71

33 Hu Y, Chen X, Pan TT, Neo KL, Lee SW, Khin ES, Moore PK, Bian JS. Cardioprotection induced by hydrogen sulfide preconditioning involves activation of ERK and PI3K/Akt pathways. *Pflugers Arch* 2008;**455**:607–16

34 Schulz R, Belosjorow S, Gres P, Jansen J, Michel MC, Heusch G. p38 MAP kinase is a mediator of ischemic preconditioning in pigs. *Cardiovasc Res* 2002;**55**:690–700

(Received May 5, 2009, Accepted October 29, 2009)