Intermittent hypoxia-induced delayed cardioprotection is mediated by PKC and triggered by p38 MAP kinase and Erk1/2.

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Abstract

Objective. We previously reported that acute intermittent hypoxia (IH) confers delayed cardioprotection against a prolonged ischemic insult in the rat, via the involvement of nitric oxide synthase and K_{ATP} channels. In the present study, we investigated the role of protein kinase C (PKC), phosphatidylinositol-3-kinase (PI3K), stress activated p38 MAP kinase (MAPK) and extracellular signal regulated kinase (ERK1/2) using selective inhibitors of these pathways. **Methods.** Adult male rats were exposed to 1-min cycles of IH (10% O_2, 40 sec) / normoxia (21% O_2, 20 sec) during 4 hrs or to normoxic cycles. 24 hrs later, isolated hearts were perfused in Langendorff mode and subjected to a 30-min global ischemia followed by 120 min of reperfusion. **Results.** Compared to normoxic conditions, IH significantly reduced infarct size (22.2 ± 2.4% vs 33.8 ± 2.6%, $p<0.05$), improved coronary flow and decreased the contracture at reperfusion. When administered before sustained ischemia, chelerythrine (a PKC inhibitor) abolished both the IH-induced reduction in infarct size (36.1 ± 4.9%) and improvement in hemodynamic parameters. In contrast, chelerythrine administration 10 min before IH, did not modify the delayed cardioprotective response. Similarly, wortmannin (a PI3K inhibitor) administration 10 min before IH was unable to block the cardioprotective effects. However, administration of SB203580 (a p38 MAPK inhibitor) and PD98059 (an Erk1/2 inhibitor), 30 min before IH abolished its delayed infarct-sparing effect (32.2 ± 3.4% and 33.9 ± 2.9% respectively). In addition, 24 hrs after IH, a significant increase in p38 MAPK and Erk1/2 phosphorylation was observed by Western blot. **Conclusion.** These results suggest that the delayed preconditioning induced by intermittent hypoxia does not involve the PI3K signalling pathway and that is mediated by PKC and triggered by p38 MAPK and Erk1/2.

Key words: intermittent hypoxia, hypoxia/anoxia, infarction, preconditioning, protein kinase C, MAP kinase.

Abbreviations

CF, coronary flow

Chel, chelerythrine

Dmso, dimethyl sulfoxide

HR, heart rate

I, infarct size

IH, intermittent hypoxia

KH, Krebs-Henseleit buffer

LVDP, left ventricular developed pressure

LVEDP, left ventricular end-diastolic pressure

MAPK, mitogen-activated protein kinase

MEK, mitogen-activated protein kinase kinase

N, normoxia

PI3K, phosphatidylinositol-3-kinase

PKC, protein kinase C

RPP, rate pressure product

V, ventricle size

WOT, wortmannin

Introduction

Intermittent hypoxia (IH) is one of the major components of obstructive sleep apnea syndrome [1] [2]. It has been extensively demonstrated both in animals [3] and humans [4] that IH is a determinant of cardiovascular morbidity. Surprisingly, a decline in mortality rates with age has been observed in OSA patients [5], which suggests a possible cardiovascular protection by IH [6]. There are several described differences between sustained hypoxia (SH) and IH. Notably, it has been observed a differential gene activation under IH or SH [7]. We have thus studied the acute effects of a model mimicking the OSA consequences as previously described [8].

Hypoxic preconditioning (PC), like ischemic PC [9] can provide delayed protection against ischemia-reperfusion injury [10, 11]. IH for 4 hrs with 10% O_2 induces cardioprotection illustrated by a reduction of infarct size in isolated rat heart [8].

The cellular pathways and mechanisms involved in delayed ischemic PC are well documented. In particular, the first demonstration of a role for PKC in delayed PC, was from Yamashita and colleagues who demonstrated that staurosporine, a PKC inhibitor, could prevent in vitro the acquisition of this phenomenon in hypoxic preconditioned rat cardiomyocytes [12]. Subsequently, it was observed in the rabbit, that the delayed protection following ischemic PC is abolished in vivo by chelerythrine (Chel), another PKC inhibitor [13]. The first aim of the present study was to assess whether the IH-induced reduction in infarct size is mediated and/or triggered by PKC (i.e.: chelerythrine treatment before sustained ischemia or prior to hypoxic PC).

Recent studies have revealed that phosphatidylinositol 3-kinase/protein kinase B (PI3K-Akt) pathway is involved in delayed myocardial PC as a triggering actor. Notably, wortmannin (WOT), a PI3K inhibitor, administration before ischemic PC prevents the reduction in infarct size in the rabbit [14]. Moreover, in a mice model, WOT administration before pharmacological PC abolished its antiapoptotic effect [15]. Therefore, the second aim of this

study was to test whether PI3K is involved in triggering the myocardial protection induced by IH.

The mitogen-activated protein kinases (MAPK) mediate various cellular responses including proliferation, differentiation and adaptation to stress [16]. Two major MAP kinase families have been studied in the triggering phase of PC: the p38 MAPK and the extracellular signal-regulated kinase (Erk1/2). The p38 MAPK signalling pathways has been shown to be implicated in both delayed ischemic [17] and pharmacological [18, 19] PC. Meanwhile, the Erk1/2 cascade has been involved in delayed hypoxic [20] and pharmacological PC [21]. Taken together, these findings led us to assess whether p38 MAPK and/or Erk1/2 activation participate to the IH-induced delayed cardioprotection.

Methods

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). Experiments were conducted on adult male Wistar rats (weight range 330-380 g) from Elevage Janvier (France) housed in controlled conditions and provided with standard rat chow.

Intermittent hypoxia protocol

Rats were exposed to intermittent hypoxia (IH) as previously described [8]. Briefly, rats were placed in Plexiglas chambers (length 28 cm, diameter 10 cm, volume 2.2 l). A timed solenoid valve was used to distribute nitrogen and air to each chamber. IH chambers were flushed with an air-nitrogen mixture for 40 sec to achieve hypoxia (10% O_2) followed by 20 sec of compressed air to restore normoxia (21% O_2). This 1-min cycle was repeated for 4 hrs. In parallel, compressed air at the same flow rate was distributed to sham chambers in order to submit sham animals to similar noise and airflow disturbances. The level of O_2 in the chambers was controlled throughout the hypoxia protocol using a gas analyzer (model ML206, AD Instruments).

Isolated rat heart preparation

Twenty-four hours after IH, the rats were anesthetized with 60 mg/kg i.p. sodium pentobarbital and treated with heparin (500 UI/kg, i.v.). Hearts were rapidly excised and briefly placed in cold (4°C) Krebs-Henseleit (KH) buffer before being mounted on the Langendorff apparatus via the aorta. The hearts were then retrogradely perfused at constant pressure (75 mmHg), with KH buffer containing in mM: NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.2 and glucose 11.0. The perfusion medium was

continuously gassed with 95% O_2 and 5% CO_2 and maintained at 37 °C and pH 7.4 as previously described [8]. A water-filled latex balloon (Hugo Sachs, n°4), connected to a pressure transducer, was inserted into the left ventricular cavity via the left atrium for pressure recording. Left ventricular end-diastolic pressure (LVEDP) was adjusted between 5-10 mmHg. Myocardial temperature was measured by a thermoprobe inserted into the left atrium and was maintained constant at 37°C. After 20 min of stabilization, no-flow global ischemia was induced by stopping the perfusion for 30 min. The hearts were kept at constant humidity and temperature (37°C) throughout ischemia and were thereafter reperfused for 120 min. Coronary flow (CF), expressed in ml/min/g, was measured periodically during the ischemia-reperfusion procedure, by collecting the effluent. Heart rate (HR), LVEDP, left ventricular developed pressure (LVDP = difference between left ventricular systolic pressure and LVEDP) and dP/dt max (maximal rate of left ventricular pressure rise) were recorded continuously (PC Lab 4S, AD Instruments).

Treatment groups

Twenty-four hours prior to global ischemia-reperfusion, all rats were submitted to either normoxia (N, 21% O_2) or intermittent hypoxia (IH, 10% O_2) during 4 hrs, and randomised into one of 16 groups (Figure 1):

Groups N (n=8) and IH (n=8) were only submitted to normoxic or IH exposure.

Groups N Chel1 (n=7) and IH Chel1 (n=7) were infused with chelerythrine 5 µM [23], 10 min before ischemia.

Groups N dmso 10 (n=7), IH dmso 10 (n=9), N WOT (n=9), IH WOT (n=7), N Chel2 (n=9) and IH Chel2 (n=8), were injected (i.p.) with dmso 1%, wortmannin 15 µg/kg [24] and chelerythrine 5 mg/kg [25] respectively, 10 min prior to N or IH.

Groups N dmso 30 (n=7), IH dmso 30 (n=8), N SB (n=8), IH SB (n=8), N PD (n=8) and IH PD (n=8), were injected (i.p.) with dmso 1%, SB203580 1 mg/kg [21] and PD98059 0,5 mg/kg [18] respectively, 30 min prior to N or IH.

Infarct size

At the end of the ischemia-reperfusion protocol, the atria were removed and the hearts were frozen at -20°C for 10 min. They were then cut into 2-mm transverse sections from apex to base (6-7 slices/heart). Once thawed, the slices were incubated at 37°C with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) for 10 min, fixed in 10% formaldehyde solution and photographed. Areas of infarcted tissue were measured using a computerized planimetric technique (Image Tool for Windows) and expressed as a percentage of total ventricular area (I/V).

Western blotting

To determine whether hypoxic PC was associated with an activation of Akt, p38 and Erk1/2 MAP kinases, hearts from additional animals (n=4 in each group) submitted to normoxia or IH were harvested 24 hrs later as described above. Hearts were homogenized in lysis buffer and centrifugated at 13 000 rpm for 20 minutes to remove nuclei and debris. The supernatant was collected and protein concentration was determined by the Pierce assay (Perbio Science France, Bezons, France). Proteins were loaded at 50-70 µg/lane. Phosphorylated states of Akt (phospho Akt; Ser 473), p38 MAPK (phospho p38; Thr180/Tyr182) and Erk1/2 (phospho-Erk; Thr202/Tyr204) as well as total levels of Akt, p38 MAPK, Erk1/2 and GAPDH were analysed by 10% SDS/PAGE with antibodies from Cell Signaling Technology (Ozyme, Saint Quentin Yvelines, France) (except for GAPDH from Santa Cruz Technology, Tebu-Bio, Le Perray en Yvelines, France). Equal loading was confirmed with GAPDH, and levels of

phosphorylated proteins were normalised to total protein levels assessed in the same samples and under the same conditions on separate membrane. Relative densitometry was computerized (Image J software).

Exclusion criteria

Only hearts with CF ≤ 20 ml/min/g and LVDP > 70 mmHg at the end of the stabilization period were included in this study.

Chemicals

Chelerythrine, wortmannin and PD98059 were from Calbiochem (VWR International, Fontenay sous Bois, France), dmso from Sigma-Aldrich (Saint Quentin Fallavier, France) and SB203580 was synthesised by Dr A. Boumendjel (DPM, UMR 5063 UJF/CNRS, Université Grenoble I, France).

Statistical analysis

Hemodynamic and infarct size data are presented as mean ± standard error of the mean (s.e.m.). Infarct size values and hemodynamic data were compared using two-way analyses of variance (ANOVA), with exposure and treatment corresponding to each factor. Post-hoc multiple comparisons were performed using Bonferroni tests. For blot analysis groups were compared using t-tests. Statistical significance was set at $p<0.05$.

Results

1- Intermittent hypoxia-induced myocardial preconditioning.

IH induced a delayed myocardial protection as shown by a smaller infarct-to-ventricle ratio (22.2 ± 2.4% in group IH vs 33.8 ± 2.6% in group N, $p<0.05$) (Figure 2a) and an improvement in functional recovery at reperfusion illustrated by higher CF and smaller LVEDP values (Figure 2b and Table 1). The stabilization values of hemodynamic parameters in N and IH groups are similar.

2- PKC is a mediator of IH preconditioning.

Infusion of chelerythrine 10 min prior to ischemia resulted in abolition of IH-induced cardioprotection with no difference in infarct size (36.1 ± 4.9% in group IH Chel1 vs 39.4 ± 5.5% in group N Chel1) (Figure 2a) nor in hemodynamic parameters (Figure 2b and Table 1) between hypoxic and normoxic groups. The stabilization values of hemodynamic parameters in N, IH, N Chel1 and IH Chel1 groups are similar.

3- PI3 kinase or PKC activation does not trigger IH preconditioning.

IH did not modify basal myocardial levels of Akt (Figure 3a bottom panel) compared to normoxic controls. The level of Akt phosphorylation in normoxic and hypoxic groups was similar. Wortmannin injection 10 min prior to N or IH induced a 1.5 fold decrease in Akt phosphorylation in both groups (Figure 3a). Wortmannin pretreatment did not modify the delayed protective effects on infarct size (19.0 ± 2.2% in group IH WOT vs 38.6 ± 4.3% in group N WOT, $p<0.05$) (Figure 3b) and functional recovery upon reperfusion (Table 2).

Likewise, chelerythrine pretreatment did not prevent the cardioprotective effects on infarct size (23.0 ± 1.8% in group IH Chel2 vs 35.0 ± 3.4% in group N Chel2, $p< 0.05$) (Figure 3b) and functional recovery (Table 3). Vehicle (dmso 1%) injection 10 min prior to N or IH had

no effect per se (Figure 3b and Table 2). The stabilization values of hemodynamic parameters in N dmso 10, IH dmso 10, N Chel2, IH Chel2, N WOT and IH WOT groups are similar.

4- p38 MAP kinase and Erk1/2 activation are triggers of IH-preconditioning.

SB203580 as well as PD98059 injection 30 min prior to IH abolished the IH-induced cardioprotective effects. Indeed, there was no difference in infarct size (32.2 ± 3.4% in group IH SB vs 33.5 ± 2.5% in group N SB and 33.9 ± 2.9% in group IH PD vs 26.8 ± 3.9% in group N PD) (Figure 4) and in coronary flow upon reperfusion (Table 3) between hypoxic and normoxic animals. Vehicle (dmso 1%) administration 30 min prior to N or IH had no effect per se (Figure 4 and Table 3). The stabilization values of hemodynamic parameters in N dmso 30, IH dmso 30, N SB, IH SB, N PD and IH PD groups are similar.

5- Phosphorylation of myocardial p38 MAPK and Erk1/2 after IH.

IH did not modify basal myocardial levels of p38 MAPK and Erk1/2 (Figures 5a and 6a, respectively) compared to normoxic control.

Twenty-four hours after IH, a 1.7 fold increase in p38 MAPK phosphorylation (Figure 5b) and a 1.5 fold increase in Erk1/2 phosphorylation (Figure 6b) were observed in the myocardium.

Discussion

This study provides the first demonstration that IH-induced delayed protection against myocardial infarction is triggered by p38 MAPK and Erk1/2 but not by PI3K and PKC. PKC, however, seems to play a role as a mediator of hypoxic PC. This is based on the observation that: 1) chelerythrine infusion before ischemia blocked the IH-induced cardioprotection, 2) wortmannin or chelerythrine injection prior to IH did not modify the cardioprotective response, 3) the MAP kinase inhibitor SB203580 and the MEK inhibitor PD98059, injected prior to IH, blocked the IH-induced cardioprotection and 4) a significant phosphorylation of both p38 MAPK and Erk1/2 was observed in the myocardium 24 hrs after IH.

In this study, we investigated the role of PKC in triggering and mediating IH-induced PC. We showed that IH-induced cardioprotection was abolished when chelerythrine was infused before ischemia. This is in accordance with other studies which have shown, using chelerythrine, a role for PKC in mediating delayed ischemic [13] and pharmacological [26] PC. However, the implication of other kinases can not be excluded since chelerythrine has been reported to affect some of them [27]. Furthermore, activation of PKC after ischemic PC is isoform selective. Notably, the epsilon isoform appears to be responsible for the development of delayed cardioprotection in the rabbit [28]. Thus assessment of the PKC isoform relevant to protection induced by IH should be of interest.

In a previous study [8], we suggested involvement of the ATP-sensitive potassium (K_{ATP}) channel, as mediator of protection, in delayed hypoxic PC. Protein kinase-catalyzed phosphorylation is an important mechanism by which the activity of ion channels, including the K_{ATP} channel, can be controlled [29]. There is evidence suggesting the importance of PKC in activating K_{ATP} channels during the protective mechanism of delayed pharmacological PC [30]. Thus an interaction between PKC and K_{ATP} in the IH-induced cardioprotection could be

possible and this could be investigated in measuring mitochondrial matrix redox potential (an index of mitoK_{ATP} channel activity) with and without a PKC inhibitor. In our previous study [8] we also demonstrated a role for NO synthase (NOS) in mediating IH-induced PC since a non specific inhibitor of NOS, L-NAME, abolished the delayed cardioprotection. We can suggest in the study presented here that PKC could play a role on NOS expression. Indeed, it is well-documented in ischemic or pharmacological PC, that activation of PKC-epsilon triggers a signalling cascade that leads to the binding of the transcription factor NF-κB to the inducible NOS (iNOS) promoter. This results in synthesis of new iNOS proteins, which leads to a preconditioned phenotype [31].

On the other hand, we showed that chelerythrine injection prior to IH did not modify IH-induced delayed cardioprotection. This is, to our knowledge, the first demonstration that the triggering phase of delayed PC is independent of PKC activation. However, this is consistent with previous studies focussing on early ischemic PC. The first one is by Przyklenk and colleagues who observed that PKC inhibition did not alter the infarct size reduction achieved by PC in the anesthetized open-chest dog [22]. Moreover, the work of Yang et al supports the idea that in rabbit heart, the trigger phase of PC does not require kinase activity, while the mediation phase is dependent on kinase phosphorylation [32].

The PI3K signalling pathway has been shown to confer protection against ischemia-reperfusion injury via the activation of Akt [33]. Kis and colleagues have shown that WOT blocked delayed ischemic PC and prevented Akt phosphorylation in vivo in the rabbit [14]. In contrast, we observed that WOT, even if preventing Akt phosphorylation, did not block the IH-induced PC. This discrepancy should be due to species differences, to the PC stimulus or to the WOT dosage used. Therefore, we can suggest that Akt phosphorylation is not involved in IH PC in the rat. This is also, to our knowledge, the first demonstration that the triggering

phase of delayed PC is independent of PI3K activation. However, this is consistent with previous studies focussing on early pharmacological PC. Notably, the adenosine-induced PC was shown to be insensitive to wortmannin [34, 35].

Several studies have reported the implication of p38 MAPK in delayed pharmacological PC [17, 18]. In accordance, we observed that SB203580, a p38 MAPK inhibitor, abolished the cardioprotection afforded by IH.

There is also evidence that hypoxia can activate p38 MAPK in several tissues including the heart [36, 37]. Notably, p38 MAP kinase phosphorylation was reported 24 hrs after delayed pharmacological PC [38]. This is also confirmed in our study since we observed a myocardial activation of p38 MAPK 24 hrs after IH.

Since SB203580 has been reported to also inhibit the stress activated protein kinase, SAPK/JNK [39], we cannot rule out a role of this kinase in the PC conferred by IH. Moreover, controversy exists as to the beneficial-detrimental role of p38 MAPK in cardioprotection [40]. Indeed persistent activation of p38 MAPK during ischemia has been shown to be deleterious for cells [41, 42]. This dual role may be explained by differential function of p38 subfamily members. Thus, it should be further investigated which p38 isoform is involved in the IH-induced delayed PC. Finally, the causal relationship between p38 MAPK phosphorylation and IH-induced cardioprotection should be assessed by studying the effect of SB203580 on p38 MAPK phosphorylation.

In contrast to the numerous reports involving p38 MAPK in delayed PC, there are few studies on Erk1/2, another member of the MAPK family. Two studies showed that PD98059 abolished delayed pharmacological PC [18, 21]. Our study is the first to show that PD98059 blocked the IH-induced PC. Additionally, we observe an increase in Erk1/2 phosphorylation 24 hrs after IH. This result is in accordance with a study showing an increase in Erk1/2

phosphorylation 18 hrs after hypoxia [35]. Like for p38 MAPK, the causal relationship between Erk1/2 phosphorylation and cardioprotection needs further investigation. Another limitation of our study is the use of inhibitors that are not fully specific (chelerythrine, SB203580), even if we used them at a concentration demonstrated in literature to have an effect in the rat.

One potential limitation of this study is that only one time point was chosen to examine the phosphorylation level of several protein kinases induced by IH. It would be important to examine the time course of phosphorylation/dephosphorylation of these protein kinases in the setting of IH PC and this is the subject of a future investigation.

In summary, this study shows that PKC, as a mediator, and p38 MAPK and Erk1/2, as triggers, participate to the IH-induced delayed PC. In a putative linear pathway linking these various actors of hypoxic PC, we would thus expect PKC to be downstream from MAP kinases because treatment with the p38 MAPK and Erk1/2 inhibitors abolished cardioprotection when administered before IH and treatment with chelerythrine only abolished cardioprotection when given before the prolonged ischemic period.

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Figure legends

Figure 1. Experimental protocol. Rats were submitted to normoxia (N) or intermittent hypoxia (IH) during 4 hrs. Subsequently, all animals were allowed to recover for 24 hrs. Then, after a 20 min stabilization period, a 30-min no-flow global ischemia followed by a 120-min reperfusion was performed in vitro. Chelerythrine was infused 10 min before ischemia (Chel1). In other groups, wortmannin (WOT), chelerythrine (Chel2) and their vehicle (dmso 10) were injected intraperitoneally 10 min prior to normoxic or hypoxic stress. Finally, SB203580 (SB), PD98059 (PD) and their vehicle (dmso 30) were injected 30 min prior to stress.

Figure 2. a- Infarct size expressed as a percentage of ventricles (I/V) assessed after a no-flow global ischemia (30 min)-reperfusion (120 min) sequence, in groups exposed to normoxia (N) or intermittent hypoxia with 10% O_2 (IH) without and with chelerythrine infusion 10 min before ischemia (groups N Chel1 and IH Chel1). *$p<0.05$, N.S: non significant using a two-way ANOVA. **b-** Effect of N, IH and chelerythrine infusion (Chel1) on coronary flow. After 20 min of stabilization, hearts were subjected to 30 min of no-flow global ischemia followed by 120 min of reperfusion. Coronary flow was collected at 5, 15, 30, 60, 90, 120 min of reperfusion. *$p<0.05$ using a two-way ANOVA.

Figure 3. **a-** Representative Western blots demonstrating basal myocardial levels of Akt and phosphorylated Akt 24 hrs after IH in rats submitted to normoxia (N) or intermittent hypoxia (IH) and injected with dmso (dmso 10) or wortmannin (WOT). Density expressed in arbitrary units (n = 4 per group). *$p<0.05$ using a two-way ANOVA. **b-** Infarct size expressed as a percentage of ventricles (I/V) assessed after a no-flow global ischemia (30 min)-reperfusion (120 min) sequence, in hearts from rats injected with dmso (dmso 10), wortmannin (WOT) or chelerythrine (Chel2) 10 min prior to N or IH. *$p<0.05$ using a two-way ANOVA.

Figure 4. Infarct size expressed as a percentage of ventricles (I/V) in hearts from rats injected with dmso (dmso 30), SB203580 (SB) or PD98059 (PD) 30 min prior to normoxia (N) or intermittent hypoxia (IH). *p<0.05 using a two-way ANOVA.

Figure 5. a- Representative Western blots of basal myocardial levels of p38 MAP kinase (top panel) and phosphorylated p38 MAP kinase (bottom panel) 24 hrs after normoxia (N) or intermittent hypoxia (IH). **b-** Ratio of phosphorylated p38 MAP kinase over total p38 MAP kinase content in N and IH groups (n=4 per group). GAPDH demonstrates equal protein loading in experimental groups. Data are expressed as a percentage of normoxic control values. *p<0.05 using a t-test.

Figure 6. a- Representative Western blots demonstrating basal myocardial levels of Erk1/2 and phosphorylated Erk1/2 24 hrs after normoxia (N) or intermittent hypoxia (IH). **b-** Ratio of phosphorylated Erk1/2 over total Erk1/2 content in N and IH groups (n=4 per group). GAPDH demonstrates equal protein loading in experimental groups. Data are expressed as a percentage of normoxic control values. *p<0.05 using a t-test.

Table 1. Hemodynamic parameters recorded in hearts from rats submitted 24 hrs earlier to normoxia (N) or intermittent hypoxia (IH) without and following chelerythrine infusion (Chel1) 10 min before ischemia.

		N	**IH**	**N Chel1**	**IH Chel1**
CF					
(ml/min/g)	*R 15*	6.0±0.2	8.5±0.9	4.4±0.3	4.6±0.4
	R 60	5.0±0.4	7.5±0.9	3.7±0.2	3.8±0.4
	R 120	4.3±0.3	6.1±0.8	2.8±0.3	2.8±0.3
			*		
LVEDP					
(mmHg)	*R 15*	71.9±5.4	63.5±7.1	65.3±4.7	71.9±5.4
	R 60	54.7±5.9	45.7±6.2	38.9±3.8	54.7±5.9
	R 120	47.0±5.4	41.7±5.2	34.3±3.7	47.0±5.4
			*		
RPP					
(mmHg/min)	*R 15*	11434±1911	10331±2198	3189±1336	2249±749
	R 60	13528±1737	17136±1757	8153±1842	11426±1996
	R 120	10803±1521	13489±926	6041±1181	7019±916

CF - coronary flow, LVEDP - left ventricular end diastolic pressure, RPP - rate pressure product. R15, R 60 and R 120 after 15 min, 60 min and 120 min of reperfusion, respectively. Data are expressed as mean ± s.e.m. $^{*}p \leq 0,05$.

Table 2. Hemodynamic parameters recorded in hearts from rats pretreated 24 hrs earlier with dmso, chelerythrine or wortmannin 10 min prior to normoxia (N) or intermittent hypoxia (IH).

		N dmso 10	**IH dmso 10**	**N WOT**	**IH WOT**	**N Chel2**	**IH Chel2**
CF							
(ml/min/g)	*R 15*	5.7±0.5	6.3±0.3	6.3±0.3	8.0±0.6	8.2±0.6	8.0±0.7
	R 60	5.0±0.6	5.5±0.3	4.8±0.2	6.9±0.5	6.6±0.5	6.8±0.6
	R 120	3.7±0.4	4.4±0.1	3.7±0.2	5.2±0.4	5.0±0.5	5.0±0.5
			*		*		
LVEDP							
(mmHg)	*R 15*	64.6±4.4	62.6±2.5	76.2±2.4	64.1±3.0	49.2±2.7	48.4±5.7
	R 60	47.0±4.9	43.0±3.0	66.5±7.4	51.3±2.3	36.7±2.3	35.9±4.5
	R 120	45.1±3.9	40.0±2.9	55.4±5.4	44.5±1.5	34.7±1.5	34.0±3.4
					*		
RPP							
(mmHg/min)	*R 15*	6787±1210	9987±1830	8916±2038	8452±2356	14604±1987	15684±3187
	R 60	14645±2463	15295±937	8243±1671	13337±1457	17679±1610	16723±2362
	R 120	10701±1261	11883±961	6009±1046	11604±1626	13055±1325	12253±1732
					*		

CF - coronary flow, LVEDP - left ventricular end diastolic pressure, RPP - rate pressure product. R15, R 60 and R 120 after 15 min, 60 min and 120 min of reperfusion, respectively. Data are expressed as mean ± s.e.m. $^{*}p \leq 0,05$.

Table 3. Hemodynamic parameters recorded in hearts from rats pretreated 24 hrs earlier with dmso (dmso 30), SB203580 (SB) or PD98059 (PD) 30 min prior to normoxia (N) or intermittent hypoxia (IH).

		N dmso 30	**IH dmso 30**	**N SB**	**IH SB**	**N PD**	**IH PD**
CF							
(ml/min/g)	*R 15*	3.8±0.3	4.9±0.2	5.6±0.3	5.7±0.4	6.2±0.6	6.8±0.5
	R 60	3.2±0.2	4.2±0.2	4.6±0.3	5.3±0.5	5.1±0.5	5.3±0.4
	R 120	2.5±0.1	3.4±0.3	3.8±0.3	4.1±0.3	4.1±0.6	4.5±0.3
LVEDP			*				
(mmHg)	*R 15*	73.1±6.2	74.1±6.0	89.4±3.8	79.1±4.9	93.1±3.4	89.2±3.0
	R 60	49.8±6.2	54.1±5.4	72.3±3.1	62.3±4.1	74.6±4.0	69.4±2.7
	R 120	40.2±6.3	46.0±5.6	63.1±3.5	55.4±4.4	67.3±3.9	61.6±2.7
RPP							
(mmHg/min)	*R 15*	2577±672	3307±866	6723±978	6127±1980	9756±1317	9954±1830
	R 60	7128±1657	8458±1076	9137±1019	12354±1374	12802±1590	12394±1348
	R 120	5803±1222	8791±1161	6785±675	8888±975	9738±1203	9770±1084

CF - coronary flow, LVEDP - left ventricular end diastolic pressure, RPP - rate pressure product. R15, R 60 and R 120 after 15 min, 60 min and 120 min of reperfusion, respectively. Data are expressed as mean ± s.e.m. $^{*}p \leq 0,05$.

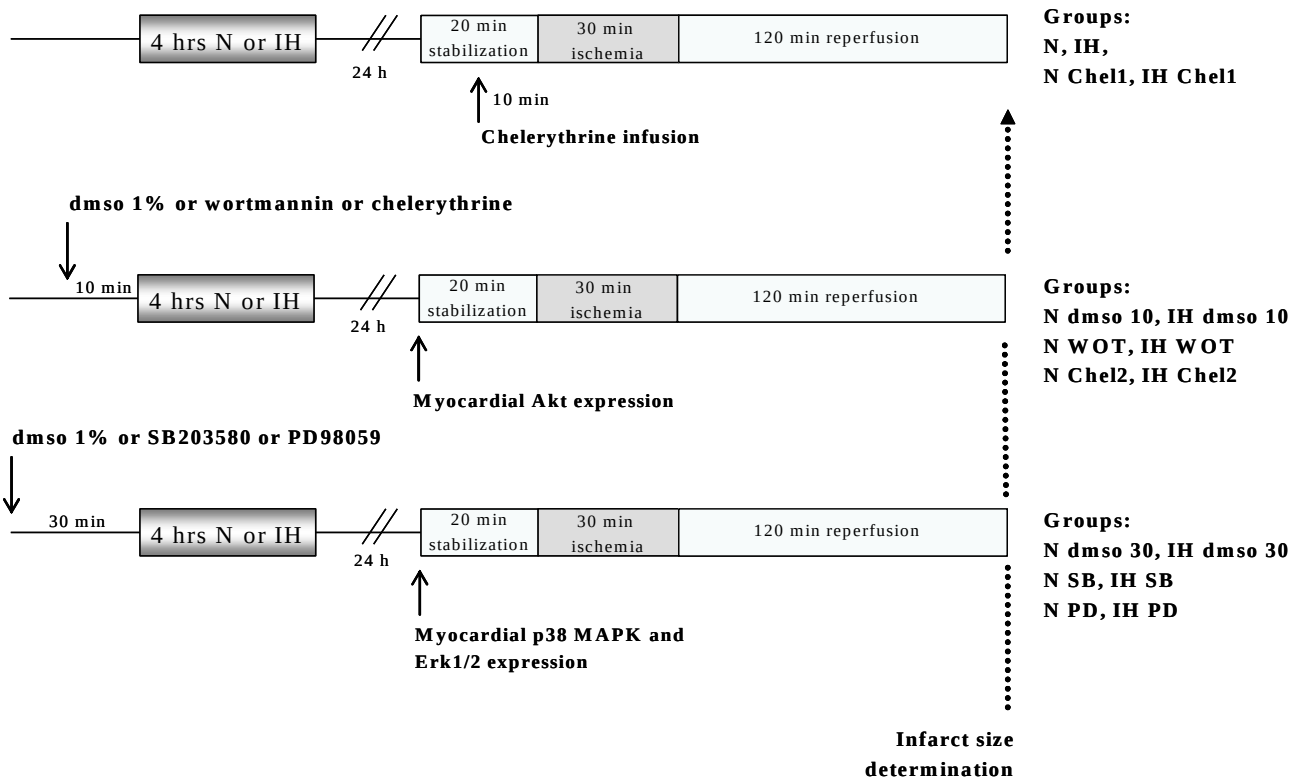
4 hrs N or IH
24 h
20 min stabilization
30 min ischemia
120 min reperfusion
10 min
Chelerythrine infusion
Groups:
N, IH,
N Chel1, IH Chel1
dmso 1% or wortmannin or chelerythrine
10 min
4 hrs N or IH
24 h
20 min stabilization
30 min ischemia
120 min reperfusion
Myocardial Akt expression
Groups:
N dmso 10, IH dmso 10
N WOT, IH WOT
N Chel2, IH Chel2
dmso 1% or SB203580 or PD98059
30 min
4 hrs N or IH
24 h
20 min stabilization
30 min ischemia
120 min reperfusion
Myocardial p38 MAPK and Erk1/2 expression
Groups:
N dmso 30, IH dmso 30
N SB, IH SB
N PD, IH PD
Infarct size determination

Figure 1.

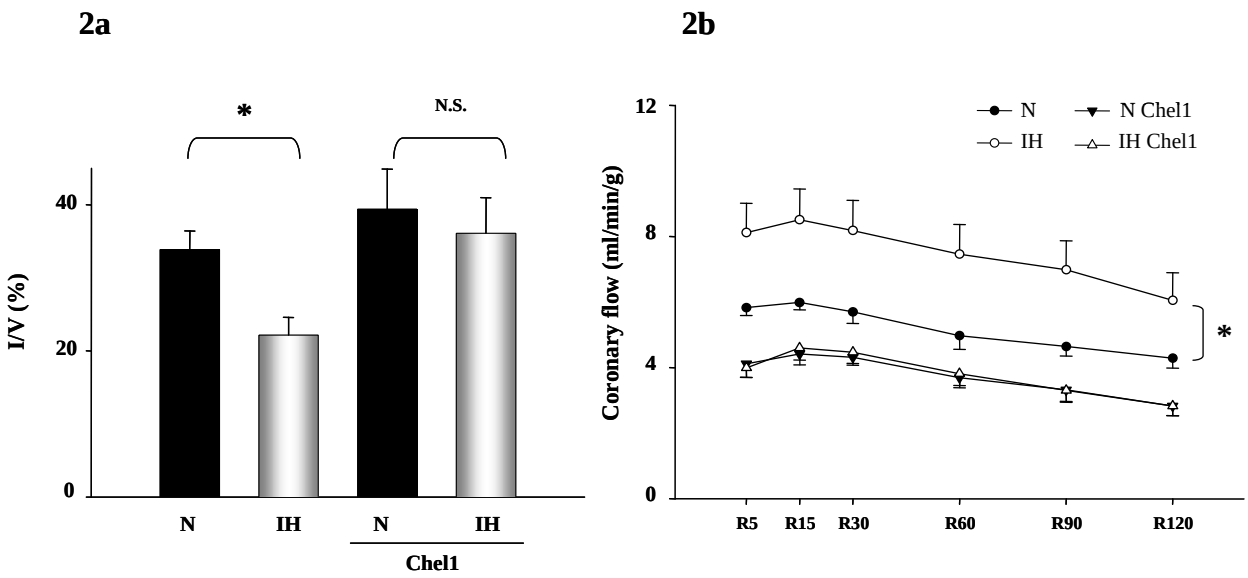
2a
2b
*
N.S.
40
20
0
I/V (%)
N
IH
N
IH
Chel1
12
8
4
0
Coronary flow (ml/min/g)
N
N Chel1
IH
IH Chel1
*
R5
R15
R30
R60
R90
R120

Figure 2.

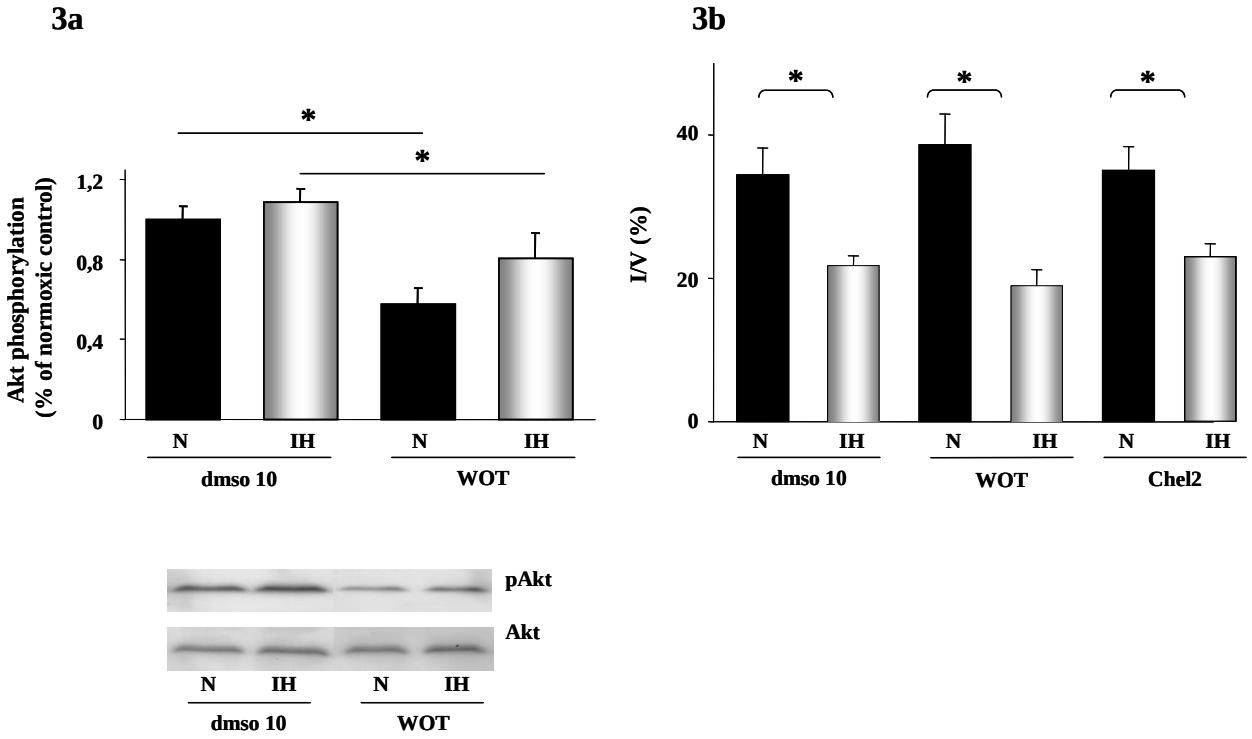
3a
3b
Akt phosphorylation
(% of normoxic control)
1,2
0,8
0,4
0
*
*
N
IH
N
IH
dmso 10
WOT
pAkt
Akt
N
IH
N
IH
dmso 10
WOT
I/V (%)
40
20
0
*
*
*
N
IH
N
IH
N
IH
dmso 10
WOT
Chel2

Figure 3.

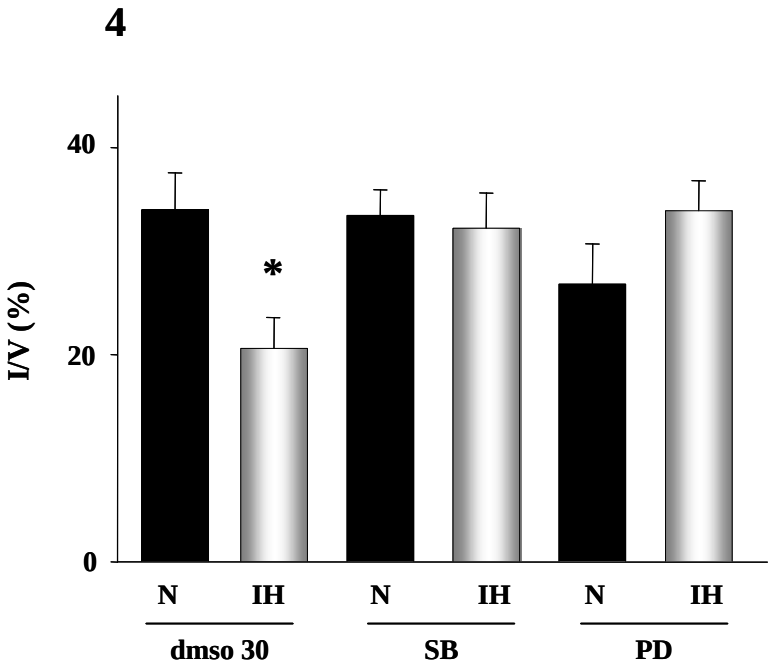

Figure 4.

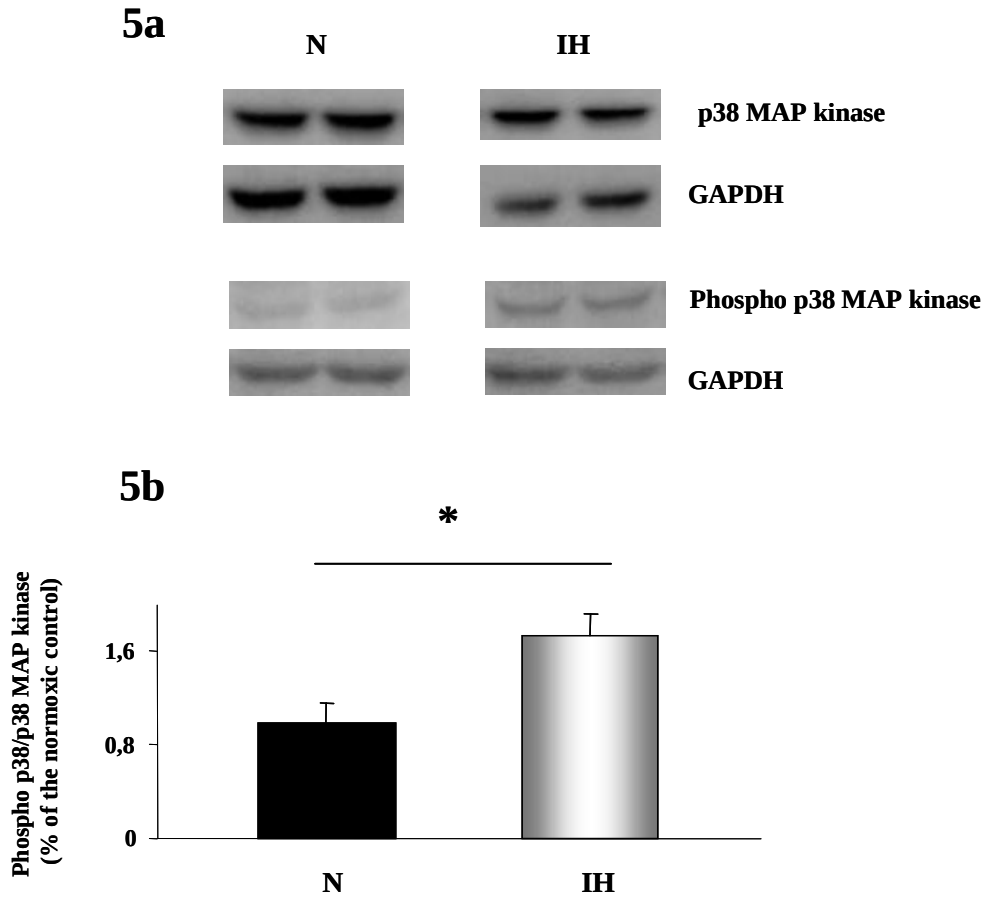
5a
N
IH
p38 MAP kinase
GAPDH
Phospho p38 MAP kinase
GAPDH
5b
*
Phospho p38/p38 MAP kinase
(% of the normoxic control)
1,6
0,8
0
N
IH

Figure 5.

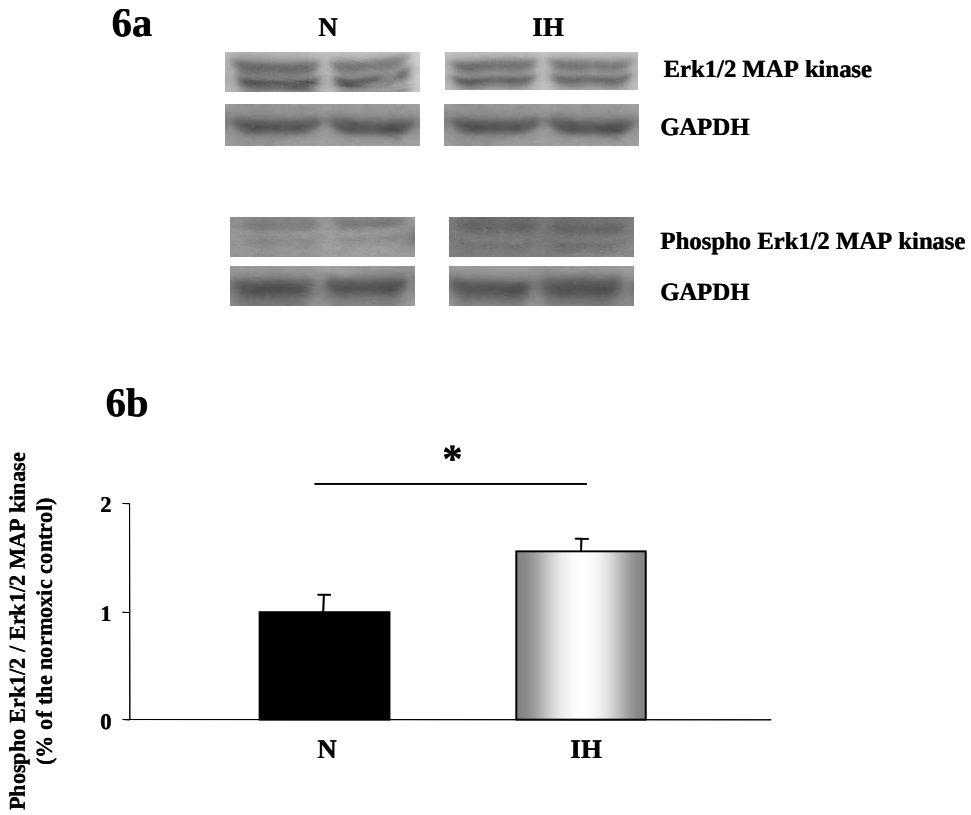
6a
N
IH
Erk1/2 MAP kinase
GAPDH
Phospho Erk1/2 MAP kinase
GAPDH
6b
*
Phospho Erk1/2 / Erk1/2 MAP kinase
(% of the normoxic control)
2
1
0
N
IH

Figure 6.