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Brief reoxygenation episodes during chronic hypoxia enhance posthypoxic recovery of LV function

Role of mitogen-activated protein kinase signaling pathways

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Abstract Children with congenital cyanotic heart defects have worse outcomes after surgical repair of their heart defects compared with noncyanotic ones. Institution of extracorporeal circulation in these children exposes the cyanotic heart to reoxygenation injury. Mitogen-activated protein kinase (MAPK) signaling cascades are major regulators of cardiomyocyte function in acute hypoxia and reoxygenation. However, their roles in chronic hypoxia are incompletely understood. We determined myocardial activation of the three major MAPKs, c-Jun NH_2-terminal kinase (JNK), extracellular signal-regulated kinase-1/2 (ERK1/2), and p38-MAPK in adult rats exposed to hypoxia (FIO_2 = 0.10) for varying periods of time. Myocardial function was analyzed in isolated perfused hearts. Acute hypoxia stimulated JNK and p38-MAPK activation. Chronic hypoxia (2 weeks) was associated with increased p38-MAPK (but not JNK) activation, increased apoptosis, and impaired posthypoxic recovery of LV function. Brief normoxic episodes (1 h/day) during chronic hypoxia abolished p38-MAPK activation, stimulated MEK-ERK1/2 activation modestly, and restored posthypoxic LV function. In vivo p38-MAPK inhibition by SB203580 or SB202190 in chronically hypoxic rats restored posthypoxic LV function. These results indicate that sustained hypoxemia maintains p38-MAPK in a chronically activated state that predisposes to myocardial impairment upon reoxygenation. Brief normoxic episodes during chronic hypoxia prevent p38-MAPK activation and restore posthypoxic recovery of myocardial function.

Key words Mitogen-activated protein kinases – MAPK – p38 – hypoxia – reoxygenation

Introduction

Infants and children with congenital cyanotic defects have their hearts chronically perfused with hypoxemic blood. These children show myocardial damage after surgical correction of their heart defects [5, 6, 12, 17, 21, 22]. Institution of cardiopulmonary bypass in these infants exposes the cyanotic heart to sudden reoxygenation. Amongst all conditions that cause myocardial injury, periods of prolonged hypoxemia

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followed by abrupt reoxygenation cause some of the most damaging and irreversible consequences. Experimental evidence suggests that immature hearts are tolerant to ischemia and reperfusion, but susceptible to reoxygenation injury during cardiopulmonary bypass without aortic clamping [11]. Clinical evidence in cyanotic infants and children undergoing surgical repair of congenital heart defects suggests that chronic hypoxemia followed by sudden reoxygenation on cardiopulmonary bypass causes free radical production and impaired postbypass contractility [5, 6, 12]. Ensuing low-output syndrome is the leading cause of prolonged and complicated courses after successful heart surgery in cyanotic infants and children [12, 17, 21, 22].

Stress and mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that are activated in response to various extracellular stimuli such as chemical stress, physical stress, radiation, and G-protein-coupled receptor activation. Three major MAPK signaling cascades include stress-activated protein kinases (SAPKs)/c-Jun N-terminal kinases (JNKs), p38-MAPK (p38α, p38β, p38γ, and p38δ isoforms), and extracellular signal-regulated kinases (ERKs). Acute myocardial hypoxia and reoxygenation stimulates MAPK activation [19]. However, the roles of MAPKs in chronic hypoxia are still incompletely understood. A recent study showed increased JNK and p38-MAPK activation in cardiac tissues from hypoxic infant rabbits and humans [23]. In the rabbit model, pups were housed in hypoxic chambers but were allowed to return to their mothers and, hence, to breathe room air for a 30-min feeding period daily [23].

Traditionally, hypoxic or hypobaric chambers that were used in previous studies required exposure to room air for a 15–30-min feeding and cleaning period daily [2, 13, 23]. To avoid this confounding factor, we have designed new normobaric hypoxic chambers that allow for daily animal care, drug injection, sacrifice, and organ recovery in the absence of any exposure to room air [3, 20]. Therefore, the myocardium is chronically perfused with hypoxemic blood. This nonsurgical model mimics physiological (high-altitude) and pathological (congenital heart defects, obstructive lung disease, anemia, microvascular disease) conditions of impaired tissue oxygenation. Unlike previous studies, we used a hypoxic perfusate for initial equilibration of the isolated hypoxic heart to avoid premature reoxygenation. Thus, our model permits to study the response of chronically hypoxemic hearts to the first exposure to high O_2 tensions. We used this model to assess myocardial MAPK activation in chronic hypoxia and subsequent reoxygenation. Since previous studies using conventional hypoxic chambers required intermittent exposure to room air, we also studied the effect of repeated brief normoxic episodes on MAPK activation in chronically hypoxic hearts.

Methods

Experimental groups

Animal experimentation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 8523, revised 1996). Animals were housed in normobaric hypoxic plexiglas chambers that allow for daily maintenance in the absence of any exposure to room air [3]. The oxygen tension inside the hypoxic chamber was continuously monitored by an O_2 electrode. We assessed MAPK activation after exposure to a hypoxic environment (fraction of inspired oxygen [FIO_2] = 0.10) for varying periods of time (Fig. 1). Sprague–Dawley male rats (5 weeks of age; purchased from IFFA-Credo, L'Arbresle, France) were subdivided into five groups (n = 6/group): (1) Normoxia (N; room air, [FIO_2] = 0.21); (2) Acute Hypoxia (AH; 1 h); (3) Sub-Acute Hypoxia (SAH; 23 h); (4) Chronic Hypoxia (CH; 2 weeks); (5) CH with intermittent normoxic Aeration (CHA; FIO_2 = 0.10 for 23 h/day and 0.21 for 1 h/day during 2 week). To assess the acute effect of a normoxic episode on MAPK activation in SAH, CH, and CHA, additional rats (n = 4/group) were exposed to room air for 1 h before the sacrifice.

Isolation of cytosolic and nuclear fractions

For sacrifice, the rat was moved to a compensation hypoxic chamber attached to the main chamber (same FIO_2) and injected with a lethal dose of sodium thiopental and 1500 U heparin IP, as described [3]. The heart was explanted and rinsed in ice-cold PBS (pH 7.4) inside the chamber. Separation of cytosolic and nuclear proteins was performed according to a published protocol [8]. The heart was freeze-clamped between steel tongs pre-cooled with liquid nitrogen. Frozen tissue was homogenized in lysis buffer solution (Hepes 10 mM, KCl 10 mM, EDTA 0.1 mM, EGTA 0.1 mM, DTT 1 mM, Triton-X100 0.5%, PMSF 0.5 mM, sodium-orthovanadate 1 mM, ß-glycerophosphate 10 mM, NaF 50 mM)

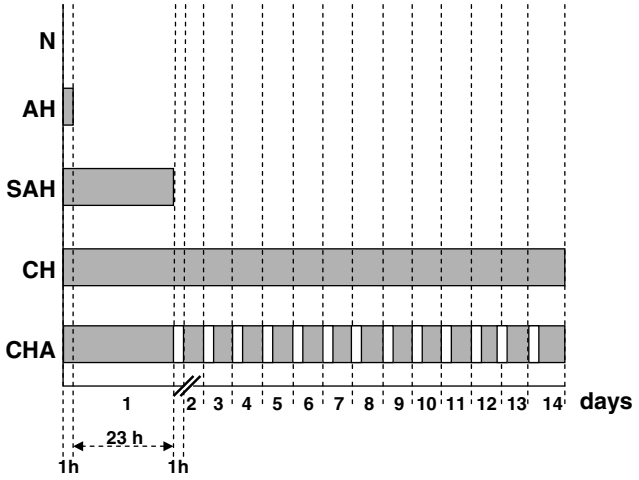

Fig. 1 Scheme of the study protocol. Experimental groups include normoxia (N), acute hypoxia (AH), subacute hypoxia (SAH), chronic hypoxia (CH); and CH with a 1-h normoxic aeration daily (CHA). Grey horizontal bars indicate hypoxia; white spaces, normoxia (the time scale is not linear)

containing Protease Inhibitor Cocktail (Roche). After incubation on ice (10 min), nuclear and cytosolic proteins were separated by centrifugation (1000 × *g*, 4°C, 5 min). Supernatant containing cytosolic proteins was transferred to a pre-cooled microcentrifuge tube, frozen in liquid nitrogen, and stored at −80°C. The pellet was incubated (4°C, 1 h) in lysis buffer solution (Hepes 20 mM, NaCl 400 mM, EDTA 1 mM, EGTA 1 mM, DTT 1 mM, PMSF 1 mM, sodium-orthovanadate 1 mM, ß-glycerophosphate 10 mM, NaF 50 mM, protease inhibitors). After centrifugation (10,000 × *g*, 4°C, 15 min), supernatant containing nuclear proteins was transferred to a cold microcentrifuge tube, frozen in liquid nitrogen, and stored at −80°C. Total protein in each fraction was measured by a modified Lowry assay using bovine serum albumin (BSA) as a standard. Purity of cytosolic and nuclear fractions was tested by Western using anti-ß-actin and anti-histone deacetylase-1 (HDAC-1) antibodies, respectively.

Western blot analysis

SDS-PAGE Western blotting was performed as follows: Proteins (80 µg) from cytosolic or nuclear fractions were heated at 95°C for 5 min, followed by electrophoresis on a 12% denaturating gel, and electroblotting onto PVDF membranes. Loading of equal amounts of each MAPK protein for each tissue sample was verified by the intensities of bands obtained with nonphospho-specific antibodies for each MAPK. Membranes were incubated with 5% nonfat dry milk in TBS-Tween buffer (1 h), followed by primary antibody (4°C, overnight), and HRP-conjugated secondary antibody (RT, 1 h). Phosphorylated MAPK protein levels were determined with phospho-specific antibodies (Cell Signaling Technologies; CST) that recognize phospho-JNK (Thr183/Tyr185), phospho-ERK1/2 (Thr202/Tyr204), or phospho-p38 MAPK (Thr180/Tyr182). Immunoblots were developed by using the LumiGlo reagent/peroxide chemiluminescent system (CST). Band intensities were quantified by using the NIH AutoExtractor-1.51 software. An extract from one normoxic heart was loaded on all blots for quantitative comparisons between blots. Data were normalized to normoxic controls and shown as fold-increases over normoxic levels.

Immunoprecipitation and in vitro kinase assays

MAPK activities were determined in nuclear and cytosolic fractions by immunoprecipitation, followed by in vitro kinase assays using specific substrates for each MAPK (n = 6/group). For determination of JNK activity, 100 µg of tissue extract was incubated (RT, 1 h) with 1 µg glutathione *S*-transferase (GST)–c-Jun coupled with glutathione beads, as described [1]. After centrifugation (10,000 × *g*, 1 min), the supernatant was removed and the beads were washed twice. Beads were resuspended (30°C, 30 min) in 20 µl kinase buffer solution (Hepes 20 mM, pH 7.5, ß-glycerophosphate 20 mM, $MgCl_2$ 10 mM, DTT 1 mM,) containing 1 µl [γ-^{33}P]ATP (3000 ci/mmol; Amersham). Samples were heated at 95°C for 5 min, followed by electrophoresis on a 12% denaturating gel. Phosphorylated c-jun was resolved by autoradiography. For determination of p38-MAPK and ERK1/2 kinase activities, 200 µg tissue extract was incubated (4°C, overnight) with anti-phospho-p38-MAPK or anti-phospho-ERK1/2, 20 µl sepharose-A beads (Amersham), and 50 µl BSA. The pellet was washed twice with lysis buffer and twice with kinase buffer solution (Tris 25 mM, pH 7.5, ß-glycerophosphate 5 mM, DTT 2 mM, sodium-orthovanadate 0.1 mM, $MgCl_2$ 10 mM) and incubated (30°C, 30 min) with 50 µl kinase buffer solution supplemented with 200 µM ATP and 2 µg ATF-2 or ELK-1 fusion proteins (CST) as p38-MAPK and ERK1/2-specific substrates, respectively. Reaction was terminated by adding 25 µl 3× SDS buffer (Tris–HCl 187.5 mM, pH 6.8, SDS 6%, glycerol 30%, DTT 150 mM, bromophenol blue 0.03%). Samples were heated at 95°C for 5 min, followed by electrophoresis on a 12% denaturating gel, and electroblotting onto nitrocellulose membrane. These were incubated (4°C, overnight) with anti-phospho-ATF-2 (Thr71) or anti-phospho-ELK-1 (Ser383) (CST), followed by HRP-conjugated secondary antibody (RT, 1 h). Immunoblots were developed with LumiGlo reagent/peroxide.

Assessment of oxidative stress and apoptosis

To assess oxydative stress and myocardial response to hypoxia, we measured plasma malondialdehyde (MDA; an index of lipid peroxidation) and nitrates–nitrites (NO_x; an index that reflects NO production), as described previously [20]. Apoptosis was determined on sections of N, AH, SAH, CH, and CHA hearts (n = 4–6/group) that were immediately processed after the sacrifice. We used terminal deoxynucleotidyl transferase-mediated dUTP-rhodamine nick end labeling (TUNEL; ApopTag Red In Situ Apoptosis detection kit; Intergen). Data are numbers of TUNEL-positive nuclei/microscopic field (mean values ± SEM) on five random fields (area: 0.037 mm^2 each) per heart section (magnification: 400×).

Isolated heart perfusion and MAPK inhibitors

Rat hearts (n = 7–11/group) were recovered inside the hypoxic chamber, perfused in a retrograde manner with a hypoxic solution, and instrumented as described [3]. After initial equilibration (30 min), hearts were reoxygenated (94% O_2) for 30 min. Rats exposed to CH received either no treatment, SB202190 or SB203580 (1 mg/kg body weight IP; Calbiochem). Both pyridinylimidazole p38-MAPK inhibitors blocking the kinase catalytic site were given 1 h before sacrifice. Inhibitory effect on p38-MAPK activity was tested by measuring phosphorylation of Hsp27, a target of p38-MAPK, in heart extracts by Western using anti-phospho-Hsp27 [15] Ab (Santa Cruz Biotech.). Normoxic rats that received either p38-MAPK inhibitor, as well as CH rats that received vehicle alone were used as controls. CHA rats either received no treatment, two doses of PD980592 (2.5 mg/kg body weight/dose IP), a potent and selective inhibitor of MAPK/ERK kinase (MEK1, the upstream activator kinase of ERK1/2) given 6 h and 30 min before sacrifice, or vehicle alone (two doses).

Statistical analysis

Statistical analysis of MAPK activation was performed by use of ANOVA. If significant, the Mann–Whitney test was used as a second step to identify significant differences between individual groups. Analysis of functional recovery in isolated hearts was performed by use of one-way ANOVA with Bonferroni's post-hoc test. Significance was set at $P < 0.05$.

Results

Blood and morphologic data

Blood and morphologic data are shown in Table 1. The O_2 level in the hypoxic chamber never increased by >1% during normal maintenance and by >2% during sacrifice. Arterial pO_2 was 34–38 mmHg in both CH and CHA versus 60–70 mmHg in N. Hematocrit, hemoglobin concentration, red blood cell counts, plasma MDA, and NO_x were increased in both CH and CHA, with a higher increase in NO_x in CHA versus CH ($P < 0.05$). CHA and CH resulted in re-

Table 1 Blood and morphological data in normoxic (N), chronically hypoxic (CH), and CH rats exposed to 1-h normoxic aeration daily (CHA)

	Controls	CH	CHA
N	9	11	8
Initial body weight, g	249 ± 18	247 ± 7	252 ± 6
Hematocrit	0.44 ± 0.01	0.69 ± 0.01[a]	0.68 ± 0.01[a]
Hemoglobin, g/l	139 ± 4	220 ± 5[a]	215 ± 2[a]
Red blood cell count, RBC/μl/1000	7.00 ± 0.15	10.26 ± 0.16[a]	9.77 ± 0.19[a]
Final body weight, g	351 ± 13	194 ± 9[a]	255 ± 8[b]
Heart weight, mg	1312 ± 43	1127 ± 35[a]	1400 ± 49[b]
(Heart weight)/(body weight), mg/g	3.83 ± 0.04	5.72 ± 0.19[a]	5.25 ± 0.12[a]
Balloon volume to increase EDP from 0 to 10 mmHg, μl	75.65 ± 6.45	81.29 ± 7.63	56.38 ± 5.69[b]
RV/LV + septum weight ratio	0.33 ± 0.02	0.59 ± 0.03[a]	0.59 ± 0.02[a]
MDA, mM	0.175 ± 0.018	0.215 ± 0.015[a]	0.213 ± 0.02[a]
NO_x, μM	2.58 ± 0.86	8.82 ± 1.50[a]	12.59 ± 0.73[ab]

Data are mean values ± SEM
[a] $P < 0.05$ versus N
[b] $P < 0.05$ versus CH
RBC, red blood cell counts; MDA, plasma malondialdehyde; NO_x, nitrates/nitrites index

duced net weight gain and weight loss, respectively. The heart weight/body weight ratio (an index of cardiac hypertrophy) and the RV/LV+septum weight ratio (an index of RV hypertrophy) were increased in both CH and CHA.

Phosphorylated (activated) MAPK protein levels

In normoxic hearts, phosphorylated JNK, ERK1/2, and p38-MAPK proteins were present in both cytosolic and nuclear fractions. In combined fractions, phospho-JNK protein levels were increased (fold) by 2.4 in AH ($P < 0.01$), 1.8 in SAH (NS), 1.5 in CH (NS), and 1.8 in CHA (NS; Fig. 2A). Phospho-ERK1/2 protein levels in combined fractions were minimally increased in AH and SAH (NS), unchanged in CH, and increased by 1.6-fold in CHA ($P < 0.05$). Phospho-ERK1/2 protein levels in nuclear fractions were increased by 1.9-fold in CHA ($P < 0.05$ and < 0.01 versus N and CH, respectively; Fig. 2C). Phospho-p38-MAPK protein levels were increased (fold) by 3.9 in AH ($P < 0.01$) and 2.4 in CH ($P < 0.05$), but marginally decreased in CHA. Phospho-p38-MAPK in nuclear fractions was increased by 4.6-fold in CH ($P < 0.01$ versus CHA; Fig. 2E). A single normoxic exposure for 1 h reduced phospho-p38-MAPK in SAH and CH by ~60% and ~30%, respectively, while phospho-JNK and phospho-ERK1/2 were essentially unaffected (data not shown).

MAPK in vitro kinase activities

Changes in specific in vitro kinase activities paralleled those in phospho-MAPK protein levels. JNK kinase activity was increased (fold) by 2.8 in AH ($P < 0.01$), 2.0 in SAH ($P < 0.05$), 1.3 in CH (NS), and 2.2 in CHA ($P < 0.05$; Fig. 2B). ERK1/2 activity was essentially unchanged in AH, SAH and CH, but was increased by 1.6-fold in CHA ($P < 0.05$; Fig. 2D). p38-MAPK activity in cytosolic fractions was increased in AH but not under the other conditions. p38-MAPK activity in nuclear fractions was increased (fold) by 4.9 in AH ($P < 0.01$), 1.7 in SAH, and 2.3 in CH ($P < 0.01$), whereas it was marginally decreased in CHA (Fig. 2F). Thus, p38-MAPK activity in nuclear fractions was reduced by a factor of 3.3 in CHA compared with CH ($P < 0.01$).

Apoptosis

Numbers of apoptotic cells were determined in both normoxic and hypoxic hearts that were processed immediately after the sacrifice (in the absence of perfusion of the isolated heart). TUNEL-positive cell counts were significantly increased in AH, SAH and CH, unlike CHA (Fig. 3).

In vivo p38-MAPK inhibition

Phosphorylation of Hsp27 was increased 3.6-fold in CH, indicating increased p38-MAPK activation, but it was suppressed by both SB203580 and SB202190, indicating effective p38-MAPK inhibition (Fig. 4).

Posthypoxic recovery of LV function

Recovery of LV function upon sudden reoxygenation was analyzed in isolated perfused hearts. Baseline measurements of LV developed pressure (LVDP), heart rate (HR), the LVDP × HR product (Fig. 5), and LV enddiastolic pressure (LVEDP; Fig. 6) were not different between groups. Posthypoxic recovery of LVDP × HR was impaired in CH but restored in CHA (Fig. 5A, E). Both SB203580 and SB202190 restored LVDP × HR in CH (Figures 5B,C). Neither p38-MAPK inhibitor affected

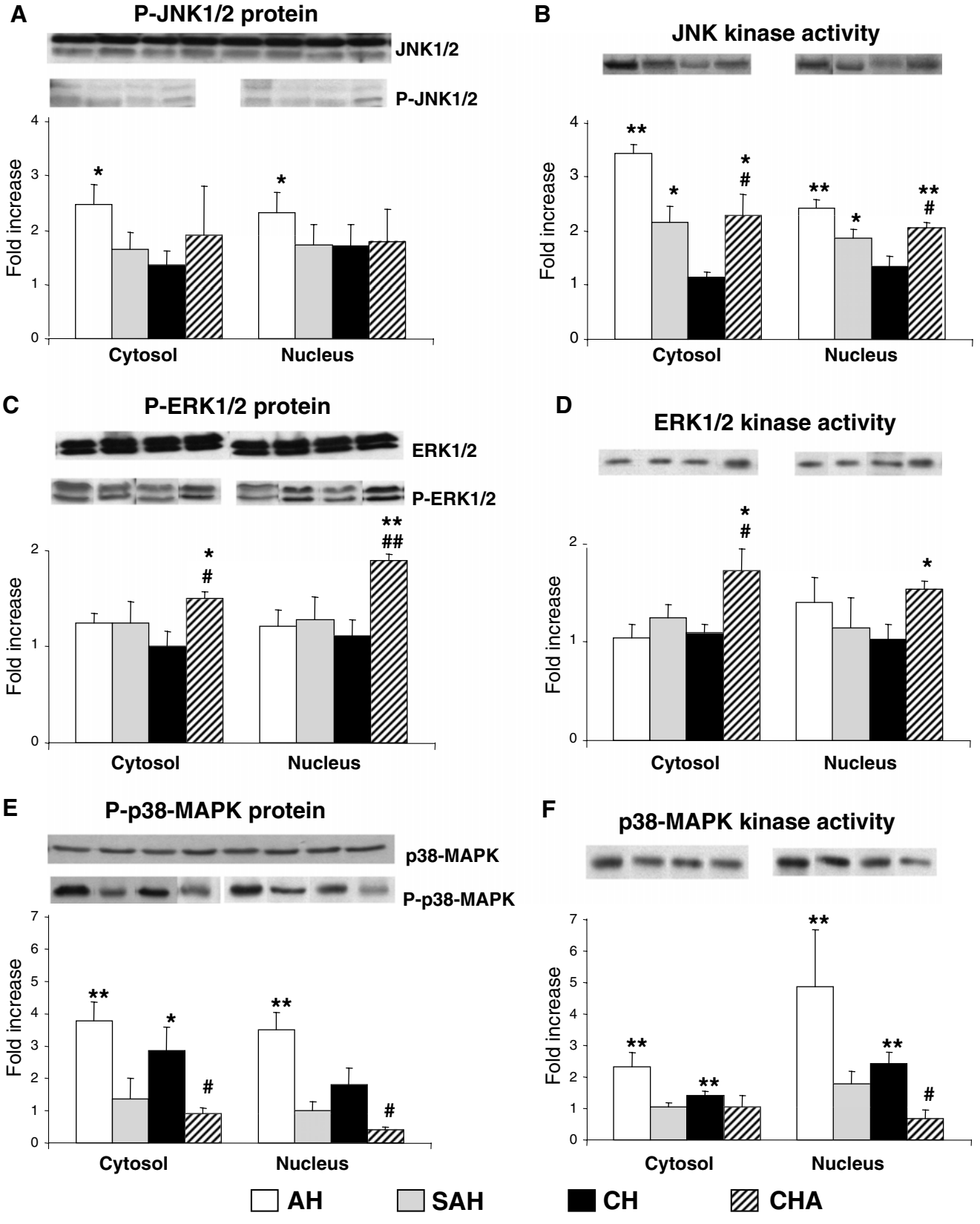

Fig. 2 Left Panels: Phosphorylated MAPK protein levels for JNK (**A**), ERK1/2 (**C**), and p38-MAPK (**E**) in cytosolic and nuclear fractions in rats exposed to AH (1 h; white), SAH (grey), CH (black), and CHA (hatched bars). Upper bands show total MAPK proteins, lower bands show corresponding phosphorylated MAPK proteins. Right panels: Specific in vitro kinase activities for JNK (**B**), ERK1/2 (**D**), and p38-MAPK (**F**). Data are fold-increases (mean ± SD) over normoxic levels. *$P < 0.05$ versus N; **$P < 0.01$ versus N; #$P < 0.05$ versus CH; ##$P < 0.01$ versus CH ($n = 6$/group)

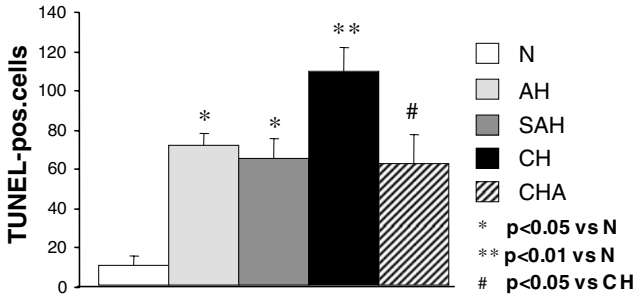

Fig. 3 Apoptosis in N (white), AH (bright grey), SAH (dark grey), CH (black), and CHA (hatched bars) hearts. Data are TUNEL-positive nuclei counts/microscopic field (mean ± SEM). *$P < 0.05$ versus N; **$P < 0.01$ versus N; #$P < 0.05$ versus CH ($n = 4$–6/group)

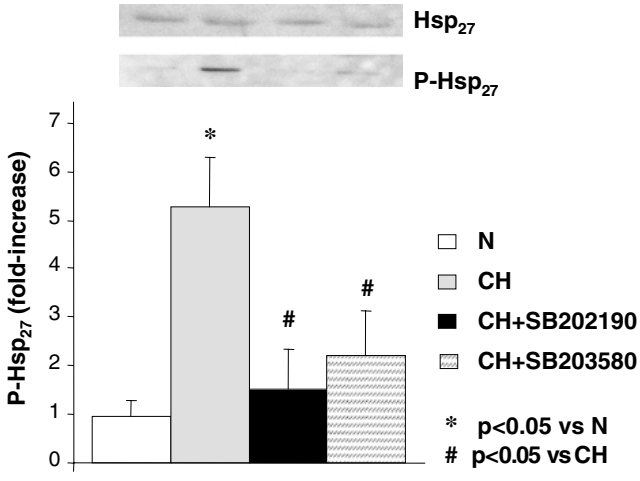

Fig. 4 Phosphorylated Hsp27 protein levels in N (white), CH (grey), CH+SB202190 (black), and CH+SB203580 (hatched bars) hearts. Data are fold-increases (mean ± SEM) over normoxic levels. *$P < 0.05$ versus N; #$P < 0.05$ versus CH ($n = 4$/group)

LV function per se in normoxic rats. PD98059 did not abolish the improvement in LVDP × HR in CHA compared with CH (Fig. 5D, E). Posthypoxic LVEDP was augmented in CH but normal in CHA (Fig. 6A, E). Both SB203580 and SB202190 reduced posthypoxic LVEDP in CH, but not in normoxic controls (Fig. 6B, C). PD98059 increased LVEDP in CHA (Fig. 6D, E); however, vehicle alone caused comparable changes (data not shown) consistent with nonspecific effects.

Discussion

The major findings in the present study are as follows: (1) MAPK signaling cascades are differentially activated in acute and chronic myocardial hypoxia in adult rats; (2) Chronic hypoxia is associated with increased p38-MAPK activation, increased apoptosis, and impaired recovery of LV function upon sudden reoxygenation; (3) Repeated brief normoxic episodes (1 h/day) during chronic hypoxia abolish p38-MAPK activation, stimulate MEK-ERK1/2 activation modestly, and fully restore posthypoxic recovery of LV function; and (4) Pharmacological p38-MAPK blockade similarly restores posthypoxic LV function.

Acute and chronic hypoxia stimulated activation of different MAPK signaling cascades in adult rat myocardium. While acute hypoxia was associated with increased activation of both JNK and p38-MAPK, chronic hypoxia enhanced p38-MAPK signaling alone. Thus, sustained hypoxia appears to maintain p38-MAPK in a chronically activated state, which potentially stimulates target nuclear factors and gene expression. This is exemplified by a ≈60% increase in the expression of c-*fos*, a p38-MAPK target gene, in chronic hypoxia (data not shown). Our results are concordant with recent data showing increased activation of both JNK and p38-MAPK in cardiac tissues from chronically hypoxic infant rabbits and humans [23], except that JNK activation was not increased in chronically hypoxic rats in the present study. Methodological factors that could account for differences in JNK activation between the two studies include species, age, and type of hypoxic chambers used. Previous protocols used conventional hypoxic chambers that required a 20–30 min normoxic period daily [2, 13, 23]. In the rabbit model mentioned above [23], infant rabbits were housed in hypoxic chambers but returned to their mothers for a 30 min feeding period daily, during which they were allowed to breathe room air [2, 23]. In contrast, we have designed novel hypoxic chambers that avoid any exposure to room air throughout chronic hypoxia (including sacrifice and heart recovery).

Chronic hypoxia was associated with detrimental effects, such as impaired posthypoxic recovery of LV function and increased apoptosis. Previous studies have suggested that the myocardium may adapt to chronic hypoxia and become more tolerant to ischemia-reperfusion injury [2, 7, 18, 23]. In a rat model, however, adaptation was confined to the right ventricle [7]. The present study focused on hypoxia-reoxygenation, rather than ischemia-reperfusion, injury, which prevents a direct comparison of our results with those obtained in ischemic models. In the clinical setting, hypoxia-reoxygenation injury occurs at institution of cardiopulmonary bypass in cyanotic infants during surgical repair of their heart defects. Experimental studies of hypoxia-reoxygenation injury without aortic clamping showed a higher magnitude of damage compared with ischemia-rep-

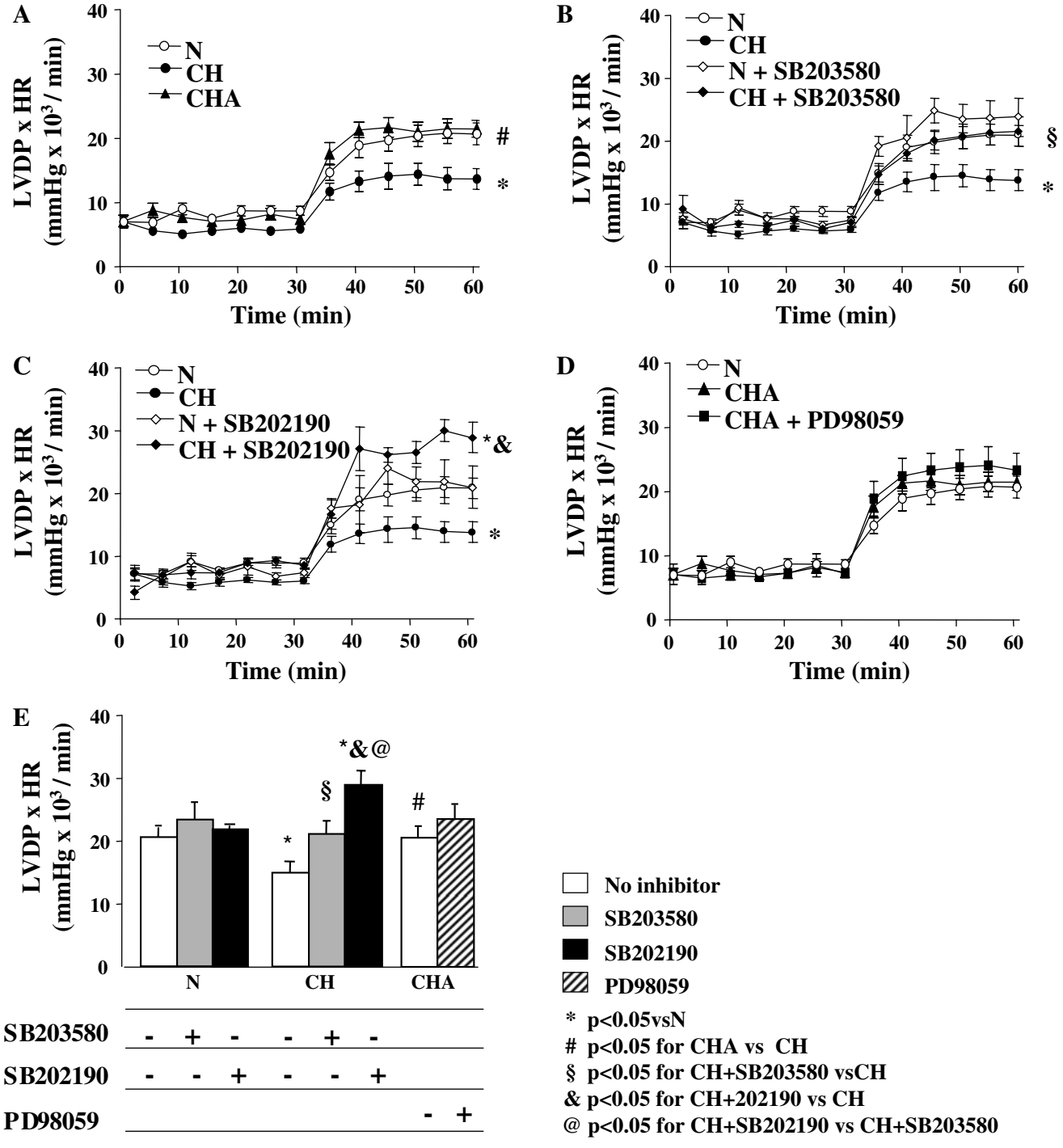

Fig. 5 Posthypoxic recovery of systolic LV function and MAPK inhibitors. (**A**) LV developed pressure (LVDP) × heart rate (HR) product during initial equilibration (starting at time 0 and ending at 30 min) and subsequent reoxygenation (starting at 30 min and ending at 60 min). The LVDP × HR product at the end of reoxygenation was decreased in CH but normal in CHA. (**B**, **C**) Effects of p38-MAPK inhibition by SB203580 and SB202190, respectively, in CH and N. Both p38-MAPK inhibitors restored posthypoxic LVDP × HR in CH, but did not induce significant changes in N hearts. (**D**) Effect of MEK1-ERK1/2 inhibition in CHA. PD98059 did not affect LVDP × HR in CHA. (**E**) Summary graph of the effects of SB203580 (grey bars), SB202190 (black bars), and PD98059 (hatched bars), as compared with no treatment (white bars), on LVDP × HR in N, CH, and CHA. Data are mean values ± SEM (n = 7–11/group)

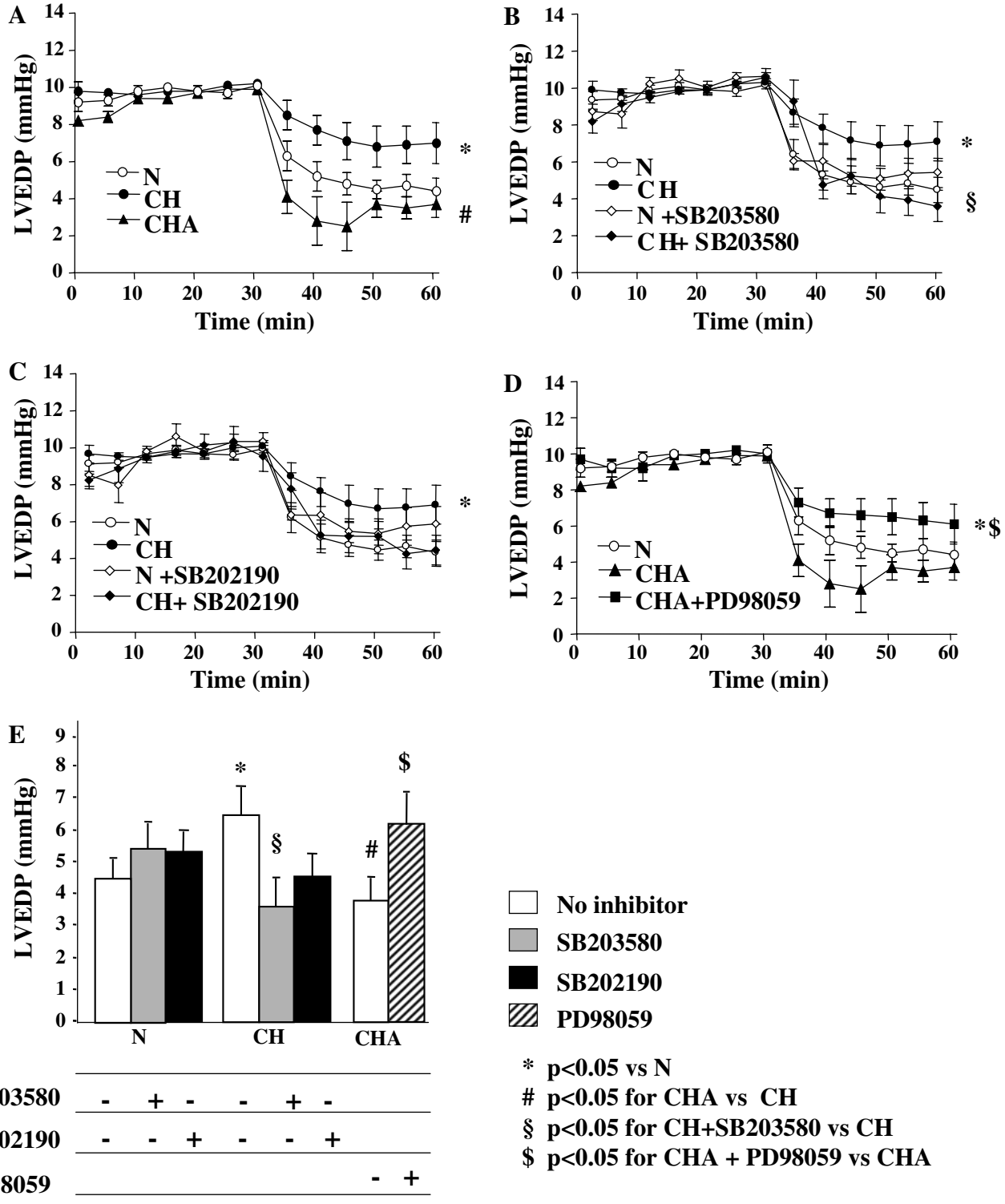

Fig. 6 Posthypoxic recovery of diastolic LV function and MAPK inhibitors. (**A**) LV end-diastolic pressure (LVEDP) during initial equilibration (starting at time 0 and ending at 30 min) and subsequent reoxygenation (starting at 30 min and ending at 60 min). LVEDP at the end of reoxygenation was increased in CH but normal in CHA. (**B**, **C**) Effects of p38-MAPK inhibition by SB203580 and SB202190, respectively, in CH and N. Both p38-MAPK inhibitors normalized LVEDP in CH but did not reduce it in N hearts. (**D**) Effect of MEK1-ERK1/2 inhibition in CHA. PD98059 increased LVEDP in CHA (similar changes were observed with vehicle alone; data not shown). (**E**) Summary graph of the effects of SB203580 (grey bars), SB202190 (black bars), and PD98059 (hatched bars), as compared with no treatment (white bars), on LVEDP in N, CH, and CHA (n = 7–11/group). Data are mean values ± SEM

erfusion injury [11]. Our results are in good agreement with a few studies in large animal models in which chronic hypoxia was induced by using surgical techniques [9, 16, 25]. In addition, they are consistent with clinical evidence suggesting that cyanotic infants and children have a higher incidence of prolonged and complicated courses after successful heart surgery compared with noncyanotic ones [12, 17, 21, 22]. Again, it is conceivable that some protective effects attributed to chronic hypoxia in models that utilized conventional hypoxic chambers were actually due to intermittent normoxic episodes during daily opening of the cages.

In the present study, exposure of chronically hypoxic rats to room air for a 1 h period daily abolished p38-MAPK, while enhancing MEK1-ERK1/2 activation modestly. In comparison, a 1 h exposure to room air following 2 weeks of hypoxia reduced phosphorylated p38-MAPK protein levels by just ≈30%. Intermittent reoxygenation for 1 h/day was paralleled by decreased apoptosis and restored posthypoxic recovery of LV function in chronically hypoxic hearts. Although cardioprotection mediated by intermittent hypoxia (4–6 h/day) was reported recently [28], it is worth noting that our results refer to intermittent reoxygenation (1 h/day) during chronic hypoxia, rather than intermittent hypoxia. The mechanism underlying the protective effect is incompletely understood. Bursts of reactive oxygen species generated during normoxic exposure after prolonged hypoxia add to oxidative stress, as evidenced by increased NO_x in the present study. It has been shown that oxidative stress induced by preconditioning contributes to the second window of protection by endogenous antioxidant enzymes in myocytes [27].

The role of p38-MAPK in hypoxia-reoxygenation injury is still controversial. p38-MAPK has been implicated in ischemic preconditioning in a pig model [24]. A number of factors, such as species, age, preconditioning, hypoxia-reoxygenation or ischemia-reperfusion injury, and timing of drug administration have been shown to influence the outcome of p38-MAPK inhibition [24]. In the present study, two different p38-MAPK inhibitors, SB203580 and SB202190, abolished p38-MAPK activation and restored posthypoxic recovery of LV function in chronically hypoxic rats, in the absence of nonspecific effects in normoxic hearts. These findings support a central role for p38-MAPK as a negative regulator of myocardial function in chronic hypoxia. They are consistent with recent data showing that p38-MAPK mediates a negative inotropic effect in cardiomyocytes [14].

At least four members of the p38-MAPK family have been identified, and its variable effects may be accounted for by the relative expression of a particular isoform. The isoforms α and β are both expressed in the heart and may have opposing effects. Ischemia activates the p38α but not p38β isoform in myocytes, a potentially detrimental pattern. The two p38-MAPK inhibitors used in the present study inhibit both p38α (SAPKα) and p38β (SAPKβ_2) [4]. The distinct roles of p38α and p38β in myocardial impairment in chronic hypoxia remain to be determined.

In the present study, we mainly focused on p38-MAPK activation as the most pronounced effect of chronic hypoxia on the three major MAPK families. Pharmacological p38-MAPK inhibition was sufficient to fully reverse the detrimental effect of chronic hypoxia on posthypoxic LV function. Thus, p38-MAPK inhibition induced by brief normoxic episodes during chronic hypoxia would be sufficient to explain the protective effect observed. Contributory roles for ERK1/2 [15] and JNK activation to this effect are possible, but have not been extensively characterized in the present study. Intermittent reoxygenation activated the MEK1-ERK1/2 signaling pathway only modestly, and the protective effect was not abrogated by PD98059, a specific MEK1 inhibitor. Moreover, intermittent reoxygenation induced in vitro JNK activity only modestly in nuclear fractions, and this was not accompanied by a parallel increase in phosphorylated JNK protein levels. Further investigations are needed to more precisely characterize the biological relevance of the modest changes in ERK1/2 and JNK activation induced by intermittent reoxygenation in the present study.

Experimental evidence for hypoxic adaptation does not appear to translate into any clinically relevant cardiac protection in children undergoing surgical repair of a cyanotic heart defect. Our data suggest that preoperative intermittent reoxygenation and pharmacological p38-MAPK inhibition might be beneficial. Parenthetically, it has been shown that breathing hyperoxic gas ($[FIO_2] = 0.80$) for 1 h induces both immediate and delayed cardioprotection in rats [26]. Regarding pharmacological approaches, a specific p38-MAPK inhibitor is being tested in initial clinical trials in rheumatoid arthritis [10]. Further studies are needed to evaluate the usefulness of these approaches in preclinical models of chronic hypoxia.

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