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The relative order of mK_{ATP} channels, free radicals and p38 MAPK in preconditioning's protective pathway in rat heart

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Abstract

Objectives: Ischemic preconditioning (PC) reduces myocardial infarction by a mechanism that involves opening of mitochondrial ATP-dependent potassium channels (mK_{ATP}), reactive oxygen species (ROS), and possibly activation of p38 mitogen-activated protein kinase (p38 MAPK). The actual order of these steps, however, is a matter of current debate. This study examined whether protection afforded by menadione, which protects by causing mitochondria to produce ROS, requires mK_{ATP} opening. In addition, we tested whether protection from anisomycin, a p38 MAPK activator, is dependent on ROS production. **Methods and Results:** Isolated, buffer-perfused rat hearts were pretreated with menadione, and infarction was assessed after 30 min of regional ischemia and 120 min of reperfusion. Menadione reduced infarction in a dose-dependent manner with an EC_{50} of 270 nM. Menadione's infarct-limiting effect was insensitive to 200 μM 5-hydroxydecanoate (5HD), an mK_{ATP} channel blocker, whereas protection by diazoxide and PC were blocked by 5HD. Anisomycin caused hearts to resist infarction and this protective effect was abrogated by SB203580, a p38 MAPK inhibitor, and 2-mercaptopropionylglycine (MPG), a free radical scavenger. **Conclusions:** These results indicate that mK_{ATP} opening occurs upstream of mitochondrial ROS generation in the protective pathway. Furthermore, protection afforded by anisomycin was p38 MAPK- and ROS-dependent.

Keywords: Free radicals; Infarction; Ischemia; Mitochondria; Preconditioning; Signal transduction

1. Introduction

Ischemic preconditioning (PC) is a cellular process triggered by brief ischemia which enables hearts to resist infarction during a subsequent prolonged period of ischemia [1,2]. The opening of mitochondrial ATP-dependent potassium channels (mK_{ATP}) contributes to PC's protection because channel openers protect against infarction whereas channel blockers inhibit protection [3–7]. There is considerable debate, however, whether mK_{ATP} channels act as signal transduction elements or as end-effectors of cardioprotection [8]. Activation of p38 MAPK may also be required for PC's protection because this kinase was specifically activated by PC, and SB203580, a p38 MAPK inhibitor, abrogated protection [9–13]. Furthermore, anisomycin, a p38 MAPK activator [14], protected in situ rabbit hearts against infarction [5]. It has been known for some time that reactive oxygen species (ROS) are involved in PC's protective effect [15,16]. The release of ROS from mitochondria reduced infarction in isolated, buffer-perfused rat hearts [17], while free radical scavengers blocked protection from PC [15]. This study examined whether there is a protective effect of low levels of ROS that is dependent upon opening of mK_{ATP} channels. That would only be seen if mK_{ATP} channels reside upstream from the ROS step in PC's signal transduction pathway. We also examined whether protection from anisomycin involved ROS.

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2. Methods

All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Infarct size studies

Male Wistar rats (250–350 g) were anesthetized with sodium pentobarbital (60 mg/kg i.p.). Hearts were surgically removed and mounted on a Langendorff apparatus as previously described [18]. Isolated hearts were perfused with warmed (37 °C) Krebs-Henseleit buffer (118.5 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 10 mM glucose) which was bubbled with 95% O_2–5% CO_2 to maintain pH 7.4. A latex balloon placed in the left ventricle measured developed ventricular pressure and a snare placed around the left coronary artery was used to produce ischemia. Fig. 1 summarizes the protocols that were used for each experimental group. All hearts were stabilized for 30 min, treated as indicated, and then subjected to 30 min of regional (index) ischemia followed by 120 min of reperfusion. PC-treated hearts received three cycles preconditioning ischemia, each consisting of 5 min of global ischemia and 5 min of reperfusion after which they were subjected to index ischemia and reperfusion. Drug-treated hearts were perfused for 20 min prior to index ischemia and reperfusion with either menadione (0.1–10.0 μM), diazoxide (50 μM), or anisomycin (100 ng/ml). The perfusion apparatus was shielded from room light with aluminum foil for the experiments with menadione. Drugs were solubilized in dimethylsulfoxide (DMSO) or saline solution as 1000-fold stock solutions and diluted as indicated prior to experiments. DMSO alone (0.2%) did not affect hemodynamics or infarct size in control studies (data not shown). Some hearts were pretreated with 5-hydroxydecanoate (5HD) (200 μM), *N*-(2-mercaptopropionyl)glycine (MPG) (1 mM), or SB203580 (10 μM) for 5 min before the above indicated drugs were perfused in combination for the next 20 min. Risk zones were marked by retrograde infusion of 1–10 μm fluorescent microspheres (Duke Scientific, Palo Alto, CA) into the aortic root after religation of the coronary artery at the end of each experiment. Hearts were frozen, cut in 1 mm slices, and incubated with 1% triphenyltetrazolium chloride in phosphate buffer for 15 min to visualize the unstained zone of infarction as previously reported [17]. Infarction was measured by planimetry and expressed as a percentage of the risk zone. Anisomycin, diazoxide, SB203580, 5-hydroxydecanoate, MPG, menadione, and laboratory reagents were obtained from Sigma Chemical Co.

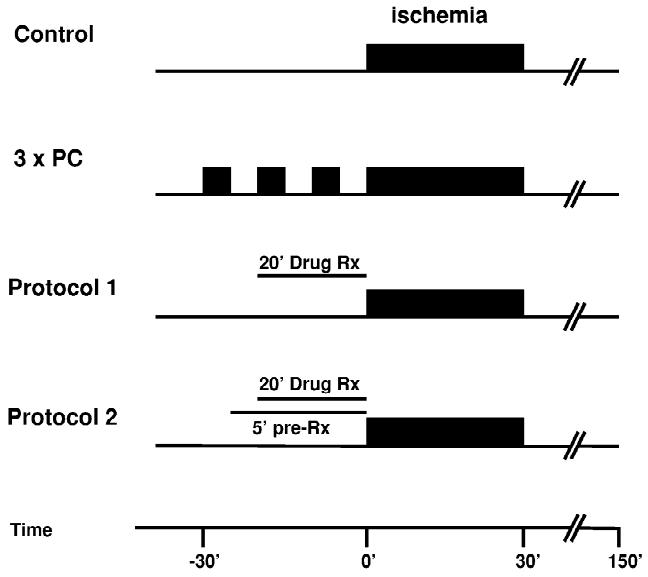

Fig. 1. Experimental protocols. All hearts were stabilized for 30 min prior to experiments. Control hearts received 30 min of regional ischemia followed by 2 h of reperfusion. PC hearts received three cycles of 5 min global ischemia and 5 min of reperfusion prior to the index ischemia and reperfusion. Protocol 1, hearts were perfused with test agents (menadione, 100 nM–10.0 μM; diazoxide, 50 μM; anisomycin, 100 ng/ml) for 20 min prior to ischemia and reperfusion. Protocol 2, hearts were pretreated for 5 min with either 200 μM 5-hydroxydecanoate (5HD), 1.0 mM *N*-(2-mercaptopropionyl)glycine (MPG), or 10 μM SB203580 (SB) prior to treatment with the drugs in protocol 1 and then drugs used in protocols 1 and 2 were co-administered for the next 20 min.

2.2. p38 MAPK activation in isolated hearts

Isolated rat hearts, affixed to a Langendorrf apparatus as described above, were treated with 3.0 μM menadione and left ventricular biopsy samples were removed at indicated times (basal, 10 min, 20 min, and 30 min). Biopsies were frozen in liquid nitrogen and homogenized following methods reported previously [17]. After homogenization in 300 μl of cold homogenization buffer (75 mM β-glycerol phosphate, 20 mM HEPES, 2 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 0.05% Triton X-100, 4 μg/ml leupeptin, 1 mM PMSF, pH 7.2), lysates were centrifuged at 13,000×*g* for 15 min at 4 °C and protein was determined in supernatants using a modified Lowry method (BioRad, #500-0116). Cytosolic samples (25 μg) were processed for SDS–PAGE/Western blotting and phosphorylated p38 MAPK was detected with a phospho-specific antibody (#9211, Cell Signaling Technology) using HRP-linked chemiluminescence (Phototope #7071; Cell Signaling Technology) following the manufacturer's specifications. p38 MAPK phosphorylation was normalized to the basal absorbance observed in untreated control samples.

2.3. H9c2 cardiac cell culture and drug treatment

H9c2 myoblast cells (ATCC, #CRL-1446) were used to evaluate the effects of menadione on p38 MAPK. Cells were plated at 5×10^5 cells/35-mm culture dish in DMEM

supplemented with 10% fetal calf serum, 5% horse serum, 0.5 unit penicillin, and 1.0 unit streptomycin and grown in a 5% CO_2 incubator at 37 °C. At 75–80% confluence, cells were washed in serum-free DMEM and incubated in 1.0 ml serum-free DMEM for 30 min at 37 °C with or without menadione at concentrations ranging from 1.0 to 10,000 nM. In some cells exposed to 10,000 nM menadione, 1 mM MPG was added 5 min prior to drug treatment. After menadione treatment, p38 MAPK activity was determined by conventional Western blotting by modification of methods described above. H9c2 cells were lysed in 300 μl of cold homogenization buffer (75 mM β-glycerol phosphate, 20 mM HEPES, 2 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 0.05% Triton X-100, 4 μg/ml leupeptin, 1 mM PMSF, pH 7.2). Cell lysates were centrifuged at 13,000×*g* for 15 min in the cold (4 °C) and protein was determined in the supernatants by a modified Lowry assay (BioRad, #500-0116). Samples (25 μg) were processed for SDS–PAGE/Western blotting and phosphorylated p38 MAPK was detected as described above with phospho-specific p38 MAPK antibody (#9211, Cell Signaling Technology) and HRP-linked chemiluminescence (Phototope #7071; Cell Signaling Technology). p38 MAPK phosphorylation was normalized to the basal absorbance observed in untreated control samples. Tissue culture supplies were obtained from GIBCO unless otherwise indicated.

2.4. Statistics

All data are presented as means±S.E.M. A one-way ANOVA with Tukey's post hoc test was performed on infarct size and baseline hemodynamics, an ANOVA with repeated measures was performed on the p38 MAPK phosphorylation data, and Student's *t*-test was performed to compare group treatment effects. Data were considered significant at the $P<0.05$ level.

3. Results

3.1. Hemodynamics

There were no significant differences in the basal heart rate, developed pressure, or coronary flow among any of the experimental groups (Table 1). Averaged group data show that menadione treatment did not decrease developed pressure more than 20%, which is comparable to effects observed during preconditioning.

Table 1
Hemodynamic data for isolated, perfused rat hearts

Group	Baseline			Preocclusion			Occlusion			Reperfusion		
	HR (beats/min)	DP (mmHg)	CF (ml/min)	HR (beats/min)	DP (mmHg)	CF (ml/min)	HR (beats/min)	DP (mmHg)	CF (ml/min)	HR (beats/min)	DP (mmHg)	CF (ml/min)
Control	329.3±20.0	113.8±3.2	13.7±0.7	–	–	–	313.3±10.0	65.2±4.1	8.7±1.0	299.2±17.8	66.2±7.3	9.7±1.2
MEN (0.1 μM)	328.0±16.2	116.4±5.0	17.2±0.5	318.0±20.8	107.4±8.2	17.2±0.5	296.0±7.5	55.6±3.2	8.4±0.7	310.0±20.7	63.8±6.7	9.2±0.8
MEN (0.3 μM)	324.0±8.1	111.8±6.1	16.2±0.7	309.0±11.2	101.0±5.6	14.8±0.5	292.0±5.8	64.6±6.1	7.4±0.7	297.0±4.9	66.2±5.5	9.4±0.8
MEN (1.0 μM)	300.0±2.6	113.3±5.1	14.7±0.4	291.7±3.0	98.3±6.5	13.7±0.5	261.7±11.9	61.0±4.3	6.5±0.4	276.7±10.2	68.6±4.6	8.0±0.4
MEN (3.0 μM)	326.0±17.7	118.4±6.0	16.0±0.4	310.0±18.4	94.2±9.5	14.0±0.3	278.0±16.6	60.2±9.1	7.0±0.4	294.0±22.4	59.0±5.2	9.4±0.5
3PC	359.2±23.9	123.3±9.6	16.3±2.0	344.0±17.8	96.2±13.3	20.3±2.3	334.2±16.9	69.8±9.6	8.5±2.0	340.0±12.4	81.2±5.8	11.2±1.7
3PC+ 5HD	334.0±12.1	102.0±5.1	16.8±0.9	314.0±16.9	68.0±4.9	21.2±1.6	246.0±17.2	50.4±6.2	6.6±0.2	295.0±8.9	51.0±6.8	10.8±0.7
MEN+ 5HD	346.0±16.6	110.8±2.8	17.2±1.3	332.0±13.6	79.0±4.8	13.8±0.9	268.0±14.6	54.0±8.1	6.8±0.9	296.0±13.2	60.8±4.3	10.0±0.3
DZ	333.3±12.5	97.3±9.6	16.0±1.0	336.6±17.4	90.0±8.7	18.5±1.6	298.3±9.1	60.0±8.5	9.2±0.7	316.7±17.6	68.5±9.9	11.3±0.7
DZ+ 5HD	368.0±21.3	102.0±6.04	18.0±1.5	384.0±15.0	96.2±4.8	18.4±1.3	320.0±15.2	53.4±3.5	8.2±1.3	378.0±18.3	51.6±3.2	11.4±1.2
DZ+ MPG	340.0±20.4	130.7±8.95	16.7±1.0	337.5±19.3	117.0±7.0	20.0±1.3	290.0±5.8	76.0±7.1	8.2±0.8	332.5±16.5	64.8±12.1	9.3±1.0
ANIS	320.0±9.3	111.7±7.0	15.0±1.1	295.0±7.2	94.7±4.9	14.3±0.8	283.4±24.7	57.5±3.4	7.5±0.9	303.3±7.1	60.7±4.6	8.8±0.8
ANIS+ MPG	352.0±21.5	103.0±9.9	15.6±0.7	318.0±18.8	95.0±10.7	16.0±1.5	278.0±20.0	61.0±6.6	7.8±0.7	298.0±26.9	63.0±5.8	8.8±0.7
ANIS+ SB	328.3±14.5	93.2±6.7	15.0±1.2	303.3±13.3	71.7±4.9	12.3±1.2	294.3±4.8	67.7±6.6	7.2±1.5	299.0±6.7	60.2±5.1	8.0±0.6
5HD	315.0±19.5	100.0±6.1	15.2±1.1	318.0±22.4	96.4±7.9	15.2±1.0	267.0±23.4	56.4±5.1	6.6±0.5	304.0±12.5	56.0±5.9	8.0±1.4
MPG	370.0±14.6	88.0±4.4	15.6±0.4	360.8±20.4	79.8±2.7	15.3±0.9	316.7±24.6	46.7±1.9	6.8±0.6	340.0±15.7	44.8±3.4	8.8±0.8

HR, heart rate; DP, developed pressure; CF, coronary flow; 3PC, three cycles of preconditioning; MEN, menadione; 5HD, 5-hydroxydecanoate (200 μM); DZ, diazoxide (50 μM); ANIS, anisomycin (100 ng/ml); MPG, *N*-(2-mercaptopropionyl)glycine (1.0 mM). Values for baseline were measured after 30 min stabilization. Values for preocclusion, occlusion, and reperfusion were measured before the 30-min ischemia, at the end of 30 min of ischemia, and at the end of 60 min of reperfusion. Values represent mean±S.E.M.

3.2. Infarct size

There were no significant differences in body weight, heart weight, or size of risk zones between any of the experimental groups (Table 2). As illustrated in Fig. 2, treatment with menadione caused a dose-dependent reduction of infarction. Infarction was 32.6±3.7% in control hearts (n=8). Treatment with 100 nM menadione slightly reduced infarction to 28.6±4.5% (n=5). Infarction was significantly reduced to 15.5±4.2%, 8.7±1.6%, and 10.9±2.7% (P<0.05) in hearts treated with 300 nM (n=5), 1000 (n=6), and 3000 nM menadione (n=6), respectively. This protective effect was attenuated at 10 μM menadione (infarction=22.3±4.5%; n=3), possibly a result of toxicity at higher doses (data not shown). These data were fit by a dose–response algorithm (Origin 6.1, Rockware, Golden, CO) which yielded an EC_{50} of 270 nM for the protective effect of menadione (inset, Fig. 2). The infarct-limiting effect of menadione was observed at much lower doses than previously reported for protection in heart tissues [19,20].

In a previous report we showed that protection induced by 3.0 μM menadione was blocked by myxothiazol, a mitochondrial electron transport inhibitor, indicating that mitochondria were the source of protective free radicals [17]. We attempted to use 2′,7′-dichlorofluorescein (DCF) fluorescence to measure ROS in isolated cardiomyocytes

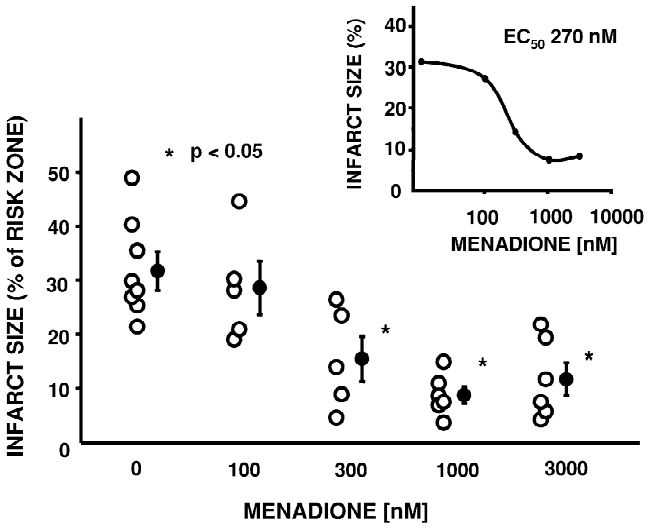

Fig. 2. The effect of menadione on infarct size in isolated rat hearts. Menadione caused a dose-dependent protection against infarction with an EC_{50} of 270 nM and saturation between 1000 and 3000 nM. Open circles are individual experimental observations, filled circles are the group means±S.E.M., and asterisks indicate significance vs. control hearts (0 nM).

[21,22], but reproducible signals could not be detected below 30 μM menadione using DCF (data not shown).

Menadione can activate p38 MAPK in various cardiac cells [17,23,24]. Therefore, in this study we evaluated the

Table 2
Infarct size data for isolated, perfused rat hearts

Group	Body weight (g)	Heart weight (g)	Risk zone (cm^3)	Infarct (cm^3)	Infarction (% of risk zone)
Control	308.8±2.1	1.37±0.10	0.280±0.02	0.090±0.01	32.6±3.7
MEN (0.1 μM)	306.0±1.9	1.35±0.02	0.309±0.02	0.089±0.02	28.6±4.5
MEN (0.3 μM)	305.0±3.2	1.35±0.03	0.307±0.01	0.047±0.01	15.5±4.2*
MEN (1.0 μM)	295.8±4.4	1.29±0.04	0.284±0.02	0.025±0.01	8.7±1.6*
MEN (3.0 μM)	313.0±12.8	1.40±0.09	0.295±0.01	0.029±0.01	10.9±2.7*
3PC	285.9±8.4	1.10±0.23	0.210±0.08	0.005±0.001	2.6±0.8*
3PC+ 5HD	308.0±6.6	1.34±0.01	0.343±0.03	0.087±0.02	24.8±2.6
MEN+ 5HD	305.0±7.7	1.30±0.06	0.285±0.02	0.015±0.009	5.2±1.2*
DZ	298.3±10.7	1.36±0.05	0.272±0.03	0.029±0.006	10.1±1.6*
DZ+ 5HD	287.0±8.2	1.26±0.05	0.285±0.01	0.061±0.009	23.4±3.8
DZ+ MPG	328.8±19.7	1.48±0.1	0.325±0.03	0.102±0.025	30.9±5.7
ANIS	272.5±8.5	1.18±0.04	0.276±0.02	0.029±0.008	10.1±4.6*
ANIS+ MPG	290.0±4.2	1.26±0.02	0.274±0.02	0.064±0.010	23.2±2.5
ANIS+ SB	335.3±14.2	1.13±0.07	0.328±0.04	0.124±0.020	38.2±4.5
5HD	290.0±6.1	1.28±0.06	0.317±0.03	0.079±0.009	24.9±1.9
MPG	299.2±2.0	1.31±0.02	0.343±0.01	0.099±0.020	28.7±5.6

For abbreviations see Table 1. Values represent mean±S.E.M. *P<0.05 vs. control infarction.

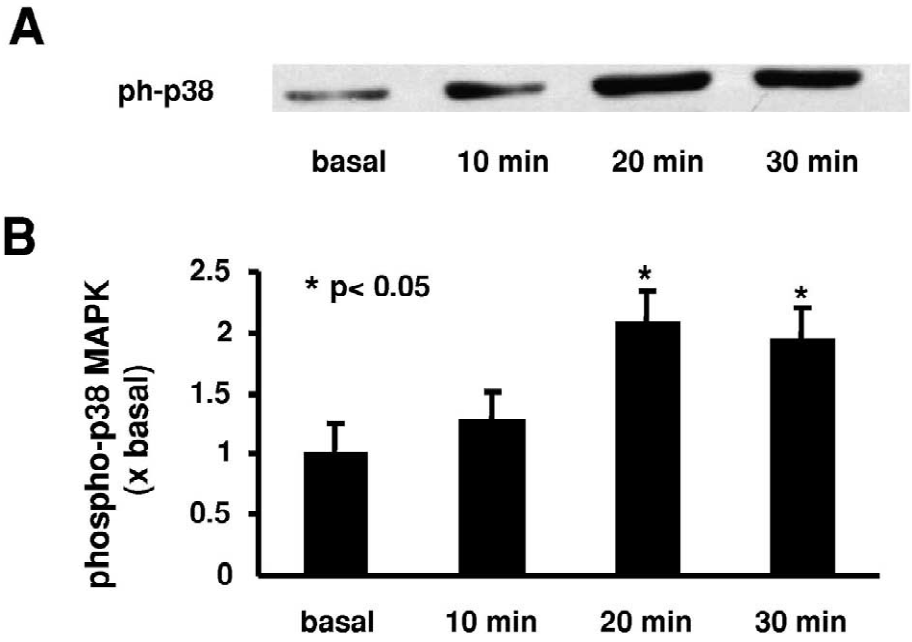

Fig. 3. Activation of p38 MAPK by menadione in isolated rat hearts. (A) Representative Western blot showing that perfusion of 3.0 μM menadione caused a time-dependent increase in p38 MAPK phosphorylation (ph-p38). (B) Group data (n=4) show that 3.0 μM menadione treatment increased p38 MAPK phosphorylation which reached a peak about twofold over basal activity after 20 min. Asterisks indicate significance vs. basal time point.

effects of 3.0 μM menadione on p38 MAPK phosphorylation in isolated rat hearts. The representative Western blot in Fig. 3A shows that 3.0 μM menadione increased p38 MAPK phosphorylation, an indicator of kinase activation, in a time-dependent manner. The group data show that perfusion with 3.0 μM menadione for 20 min increased p38 MAPK activity significantly to a level that was 207±40% over basal activity in isolated rat hearts (Fig. 3B). The dose–response relationship of menadione's activation of p38 MAPK was further investigated in H9c2 cardiac cells. Fig. 4A is a representative Western blot showing a dose-dependent activation of p38 MAPK by menadione in these cells. The average of five experiments for each group of H9c2 cells is shown in Fig. 4B. Note also that p38 MAPK activation by 10 μM menadione in H9c2 cells could be blocked by 1.0 mM MPG.

Experiments were performed in isolated rat hearts to evaluate whether mK_{ATP} channel opening contributed to menadione's protective effect. Fig. 5 shows that 5HD (200 μM), an mK_{ATP} channel blocker [7,25], did not block protection by 3.0 μM menadione. Infarction was 5.2±1.2% (n=5) and not significantly different from that in hearts treated with menadione alone (10.9±2.7%). PC and treatment with 50 μM diazoxide reduced infarction significantly, to 2.6±0.8% (n=7) and 10.1±1.6% (n=6), respectively. Protection was sensitive to 200 μM 5HD, as infarction increased to 24.8±2.6% (n=5) in PC and 23.4±3.8% (n=5) in diazoxide-treated hearts.

The mechanism of p38 MAPK activation by anisomycin is not fully understood. To examine this issue further, we treated isolated hearts with 100 ng/ml (~300 nM) anisomycin. As shown in the representative Western blot in Fig. 6A, perfusion with anisomycin caused a time-dependent increase in p38 MAPK phosphorylation which peaked after 20 min. Furthermore, as shown in Fig. 6B 100 ng/ml anisomycin was significantly protective in isolated rat hearts (infarction=10.1±4.6%; n=6) compared to control hearts. A role for p38 MAPK activation was further confirmed in hearts pretreated for 5 min with 10 μM SB203580, a widely used p38 MAPK inhibitor [12,13,17,26]. Pretreatment with SB203580 prior to anisomycin treatment blocked protection, increasing infarction significantly to 38.2±4.5% (n=6) when compared to anisomycin-treated hearts. We next examined whether anisomycin's effect might involve generation of ROS. Anisomycin's protection was significantly attenuated in hearts pretreated with 1.0 mM MPG for 5 min (infarction=23.2±2.5%; n=6) as shown in Fig. 6B. These results compare with MPG's attenuation of the protection afforded by diazoxide (infarction=28.9±5.7%; n=5), indicating that anisomycin's protection was due, at least in part, to release of ROS.

4. Discussion

This study demonstrates that the protective effect of menadione does not require mK_{ATP} channel opening which is an indication that the channels are upstream of menadione's site of action. That contrasts with the effects of PC and diazoxide which were blocked by 5HD. Menadione disrupts electron transport at site III in the

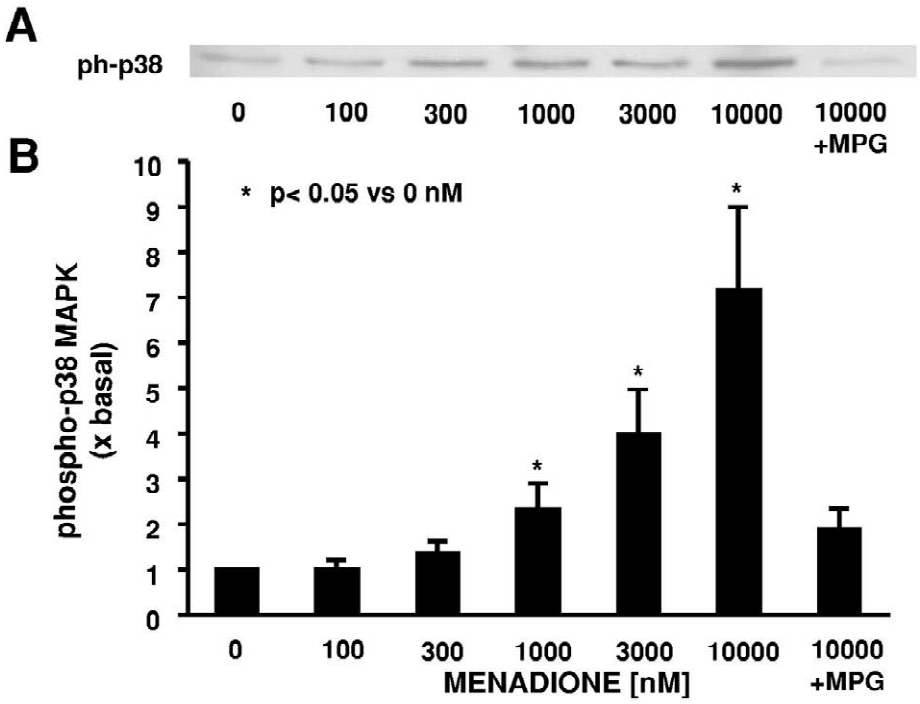

Fig. 4. Activation of p38 MAPK by menadione in H9c2 cells. (A) Representative Western blot showing that 30 min of treatment with menadione caused a dose-dependent activation of p38 MAPK. The activation of p38 MAPK by 10 μM menadione was blocked by 1.0 mM MPG. (B) Group data (n=5) indicate that menadione's threshold for p38 MAPK activation was ~300 nM and activation was sensitive to free radical scavenging by MPG.

inner membrane of mitochondria leading to the production of mitochondrial ROS [17,19,20]. Menadione may cause other effects, however, Bellomo et al. [27] reported that menadione inhibited glutathione reductase thus causing increased levels of GSH. Since that effect required a concentration of menadione greater than 25 μM, while protection was seen below 3.0 μM in the present study, it seems unlikely that altered GSH levels contributed to our findings. Menadione also causes sulfhydryl arylation in platelets [28], but that required 250 μM, which was again much higher than concentrations that we used. Moreover, menadione's protection against infarction could be blocked by myxothiazol, a site III mitochondrial inhibitor, and by MPG, a free radical scavenger, indicating the involvement of mitochondrial ROS [17]. McCormick et al. [29] reported that menadione was photochemically activated and therefore our experiments were performed with light-shielding. Furthermore, 400 μM menadione had to be exposed to 340 nM (UV) light for 60 min to observe photoactivation, while our studies used low concentration of menadione and low levels of visible light. We, therefore, conclude that menadione most likely protects the hearts by inducing ROS generation from mitochondria.

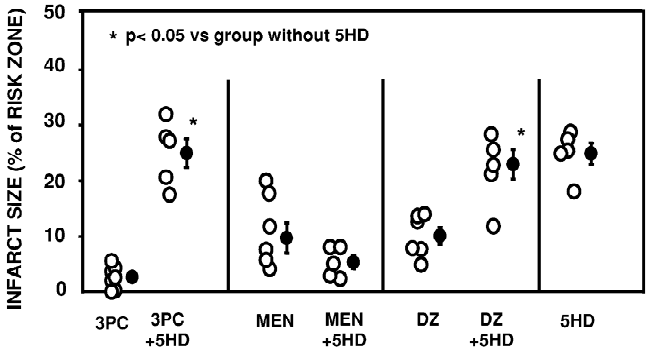

Fig. 5. The effect of mK_{ATP} channel activity on protection. The protective effect of menadione (3.0 μM) was not sensitive to 200 μM 5HD. The protection afforded by 50 μM diazoxide and PC were significantly blocked by 200 μM 5HD.

The possibility that ROS act as signaling molecules in myocardial tissues has recently gained considerable attention [21,24,30–35]. Yao et al. [21] showed that ROS were generated by acetylcholine which then protected chick cardiomyocytes against simulated ischemia in an mK_{ATP}-dependent manner. Cohen et al. [32] found that many G_i-coupled receptor systems mimic preconditioning through a ROS-dependent mechanism in the rabbit heart. While the mechanism of this protection is unknown, we speculate that mK_{ATP} channel opening releases mitochondrial ROS which are then capable of activating p38 MAPK to mediate protection. Interestingly, Carroll et al. [33] observed that 30 μM diazoxide evoked ROS which protected cultured human atrial myocytes against simulated ischemia–reperfusion injury. Furthermore, scavenging free radicals with *N*-acetylcysteine blocked diazoxide's ability to improve post-ischemic left ventricular function in rats [34]. Menadione treatment can be detrimental in cardiac

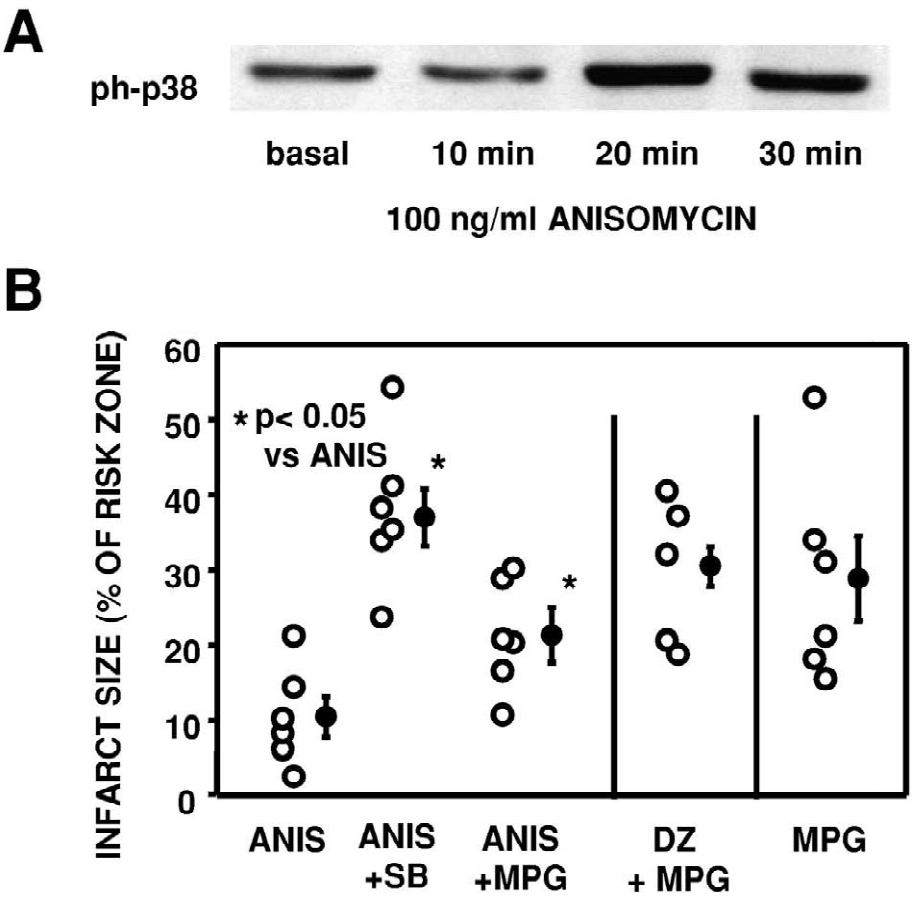

Fig. 6. The effect of anisomycin in isolated rat hearts. (A) Representative Western blot showing that perfusion of 100 ng/ml anisomycin caused a time-dependent activation of p38 MAPK which peaked after 20 min. (B) Isolated rat hearts treated with 100 ng/ml anisomycin (ANIS) for 20 min were protected against infarction. Pretreatment with 10 μM SB203580 (SB) or 1 mM MPG for 5 min significantly blocked the protective effect of anisomycin. MPG also significantly blocked the protection afforded by 50 μM diazoxide.

cells [19,20], but these effects were seen at higher doses than we used in this study. Since ROS production in isolated rat cardiomyocytes was not detectable below 30 μM menadione with DCF, we suspect that the level of mitochondrial ROS required for protection is quite low or confined to a small microcompartment. The protective effect of menadione diminished at 10 μM, as might be expected if higher levels of ROS were toxic in cardiac cells. This biphasic effect has been documented before. Turner et al. [23] reported that menadione levels up to 20 μM caused resistance to apoptosis while this protective effect disappeared above 40 μM in H9c2 cardiac cells. Interestingly, low doses of the ROS generator bilirubin protected neurons while high doses were toxic [36], and low levels of ROS enhanced survival of human aortic smooth muscle cells while higher levels were toxic [37].

Menadione can activate p38 MAPK in many cell types including H9c2 cardiac cells [23,24]. Treatment with menadione (5–40 μM) for 4 h caused the activation of p38 MAPK and c-jun NH_2-terminal kinase (JNK) in H9c2 cells [23]. Our studies indicate that p38 MAPK is activated by brief exposure to lower doses of menadione in H9c2 cells. Furthermore, low doses of menadione (1–3 μM) caused a rapid protective effect mediated by p38 MAPK in isolated rat hearts. The mechanism of p38 MAPK's protection is not completely understood. Studies in CCL39 lung epithelial cells indicate that p38 MAPK activation causes phosphorylation of HSP27, a downstream target of p38 MAPK associated with stress fiber stabilization, which mediates resistance against oxidative stress [38,39]. As shown in Fig. 7, mitochondrial ROS could activate p38 MAPK and stabilize stress fibers in a similar manner, thereby conferring resistance to osmotic swelling and attendant damage associated with ischemia in the heart. By contrast, some studies have shown that activation of p38 MAPK may be detrimental in the heart [40–43]. Schneider et al. [43] observed that SB202190, a p38 MAPK inhibitor, reduced infarct size and enhanced ventricular function in rat hearts subjected to ischemia. Saurin et al. [44] also reported that inhibition of p38 MAPK reduced cell death in rat ventricular myocytes subjected to simulated ischemia. While the reason for differences between the latter findings and the present results and others previously documenting a protective action of p38 MAPK [9–13] remains unclear,

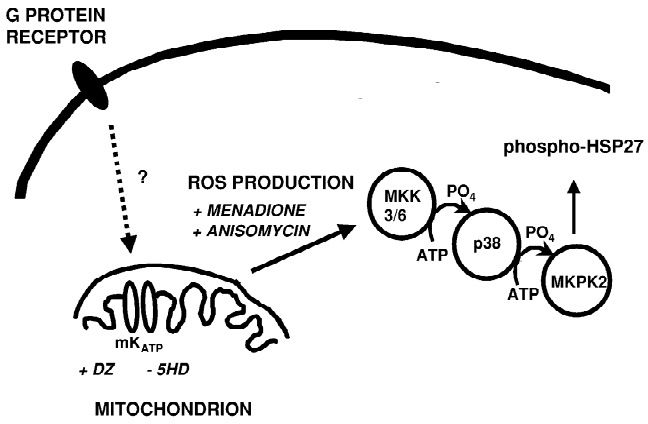

Fig. 7. Proposed mitochondrial ROS signaling pathway. We suggest that G-protein coupled receptor stimulation causes mK_{ATP} channel opening and subsequent release of mitochondrial ROS. Mitochondrial ROS release, which can be mimicked by diazoxide (DZ) and inhibited by 5-hydroxydecanoate (5HD), activates the p38 MAPK (p38) cascade. The activation of p38 MAPK then activates MAPKAPK2 (MKPK2), which then phosphorylates HSP27 to cause stabilization of stress fibers during ischemia.

recent evidence suggests that p38 MAPKα activation may be coupled to apoptosis whereas p38 MAPKβ activation may be coupled to protective cellular responses [44–48].

Isolated rat hearts were protected by 100 ng/ml anisomycin (~300 nM) and SB203580 abolished this effect in this study. While SB203580 is a widely used p38 MAPK inhibitor, other effects have been reported including JNK inhibition and phospholipase A_2 (PLA_2) activation [49,50]. There is no evidence that PLA2 is involved in PC's mechanism, but JNK has been implicated. Thus we cannot eliminate the possibility that SB203580 may have acted on JNK rather than p38 MAPK. The mechanism of anisomycin's activation of p38 MAPK is not known. It is believed to cause ribotoxic stress by disrupting ribosomal translation and inhibiting protein synthesis. Ribotoxic stress, however, is seen following prolonged exposure (>6 h) with micromolar concentrations of anisomycin [51]. By contrast, we see p38 MAPK activation and cardioprotection after brief treatment with 500-fold lower doses of the drug. Furthermore, anisomycin's protection was sensitive to MPG, suggesting a role for ROS. Recently, 10 ng/ml anisomycin (~30 nM) produced ROS which activated p38 MAPK in PC12 cells [52]. Our results are the first to suggest that anisomycin's protection, like that of menadione, is mediated by the production of ROS in cardiac cells. This observation raises several interesting possibilities. Firstly, perhaps it is activation of p38 MAPK that causes ROS production rather than the other way around. But that would not be compatible with our observation that generating ROS directly with menadione caused p38 MAPK activation in the H9c2 cells and that MPG could block that activation. The second explanation would be that anisomycin may produce ROS as a non-specific side effect which is unrelated to p38 MAPK blockade and that it is the ROS rather than the p38 MAPK activation that protected the hearts. That, however, would not explain why the p38 MAPK inhibitor SB203580 blocks anisomycin's or menadione's protection. The most likely explanation then would be that anisomycin activates p38 MAPK by causing ROS production in the cell. Clearly this is an issue for further study.

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