Acidosis-induced p38 MAPK activation and its implication in regulation of cardiac contractility[1]

Ming ZHENG[2], Rong HOU, Rui-ping XIAO

The Institute of Cardiovascular Sciences, Peking University, Beijing 100083,China

KEY WORDS mitogen-activated protein kinases; myocardium; ischemia; acidosis; contractility

ABSTRACT

AIM: To determine the possible role of pH in mediating activation of p38 mitogen-activated protein kinase (MAPK) and the consequent function of activated p38 MAPK in regulating cardiac contractility. **METHODS**: Adult rat cardiomyocytes were isolated and cultured. Low pH media was used to induce intracellular acidosis and contraction of single cardiomyocyte was measured. **RESULTS:** Phosphorylation of p38 MAPK was increased during ischemia, and pH_i was decreased. Intracellular acidosis activated p38 MAPK to a similar level as ischemia. Inhibition of p38 MAPK activation by SB203580, a specific inhibitor of p38 MAPK, reversed acidosis-mediated reduction of myocyte contractility. **CONCLUSION:** In adult rat cardiomyocytes, intracellular acidification activated p38 MAPK and decreased cardiac contractility. Pretreatment of cardiomyocytes with SB203580 completely blocked p38 MAPK activation and partially reversed acidosis-mediated decline of cardiac contractility.

INTRODUCTION

The pH of the internal milieu in mammals is strictly regulated under normal conditions. The decrease of extracellular pH (pH_o) may result in metabolic or respiratory acidosis and represents a significant environmental stress to cells. These acid conditions have dramatic effects on cell functions including cell survival and cell death[1,2]. Measurements of intracelluar pH (pH_i) have shown a correlation between intracellular acidification and the onset of apoptosis. In cell lines such as T lymphocytes, a population of cells with a low pH_i was observed after deprived of growth factors or stimulated by stress such as UV radiation[3,4].

A large number of studies has reported the decrease of pH_i during ischemia. In cerebral ischemia the pH_i falls by 0.5-1 unit[5]. The mechanism for the maintained decrease in pHi has not been studied fully but has generally been assumed to be the activation of glycolysis stemming from the lowered ATP. The pH fall usually correlates with increased lactate concentration as blood glucose is increased, however, there are indications that the regulation is more complex. The reduction in pH during ischemia makes a major contribution to development of damage. In cats, completely prevention of the extracellular acidification during ventricular ischemia by using an alkaline buffer reduced the infarct volume by 40 %[6]. Most of the existing data are consistent with the hypothesis that the fall in pH is damaging, however, contradictory findings have been reported. *In vitro*, artifactually reducing pH is protective against ischemia[7,8], as yet the underlying mecha-

[1] Project supported by the National Natural Science Foundation of China (No 30100215) and (30225036), Peking University 985 Project, and the State Key Basic Research and Development Program of China (973) (G2000056906).
[2] Correspondence to Dr Ming ZHENG, MD, PhD.
Phn 86-10-8280-1146. Fax 86-10-8280-2769.
E-mail zhengm@bjmu.edu.cn
Received 2003-09-09 Accepted 2004-04-08

nisms of the protection is not completely known.

It was reported that mitogen-activated protein kinase (MAPK) pathways of mammalian cells were stimulated by low environmental pH treatment[9]. p38 MAPK, also called a stress-activated protein kinase, is generally reported to be involved in the onset of cardiac hypertrophy and cell death in response to *in vivo* pressure overload or ischemic/reperfusion injury[10,11]. As stated previously, ischemia is always consistent with low pH, and a large body of studies in both whole hearts and isolated cardiomyocytes also reported the activation of members of the MAPKs during ischemia[12,13]. However, the effects of low pH on p38 MAPK pathways are not yet known. More recently, our studies demonstrated that activation of p38 MAPK also was involved in regulation of cardiomyocytes contractility[14,15]. Therefore, in this study we investigated whether p38 MAPK was activated during the ischemia in adult rat cardiomyocytes and the role of activated p38 MAPK in low-pH mediated decrease of cardiac contractility.

MATERIALS AND METHODS

Isolation of adult rat ventricular myocytes Single cardiac myocytes were isolated from 2-4 month-old rat hearts by a standard enzymatic technique, as described previously[16]. Briefly, rat hearts were quickly removed under sodium pentobarbital anesthesia and retrogradely perfused with a low Ca^{2+}, collagenase bicarbonate buffer solution (36 ºC, pH 7.4). When the heart became soft, the perfusion was terminated, the left ventricle was mechanically dissociated and cells were suspended in Hepes buffer solution consising of (mmol/L): $CaCl_2$, 1.0; NaCl, 137; KCl, 5.0; dextrose, 15; $MgCl_2$, 1.3; NaH_2PO_4, 1.2; Hepes (5-*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid), 20; pH was adjusted to 7.4 with NaOH.

Cell contraction measurement Cells were placed on the stage of an inverted microscope (Zeiss, model IM-35), perfused with Hepes-buffered solution, and electrically stimulated at 0.5 Hz at 23 ºC. Cell length was monitored by an optical edge-tracking method using a photodiode array (model 1024 SAQ; Reticon) at a time resolution of 3 ms. Cell contraction was measured by the percent shortening of cell length after electrical stimulation, as previously described[16].

Cell pelleting model (simulated ischemia and cellular injury) A cell-pelleting model of ischemia was used, as previously described[17]. Briefly, cell suspension (0.5 mL) was centrifuged at a low speed 1200×*g* for 60 s into a pellet, supernatant was removed except of a thin fluid layer above the pellet. Mineral oil 0.2 mL was added on the top of the pellet to prevent gaseous diffusion. After 1 h of pelleting, cells were subjected to measurements of pH_i and p38 MAPK activation.

Measurement of intracellular pH Intracellular pH was measured by a fluorescent indicator, SNARF-1 (carboxy-seminaphthorhodafluor-1) (from Molecular Probe), as described previously[15]. Briefly, cells were incubated with SNARF-1 (5 μmol/L) for 15 min, washed with fresh Hepes-buffer, and the emission spectrum was measured. The absolute values of intracellular pH in individual myocytes were obtained from a standard pH curve using *in vitro* calibration.

p38 MAPK phosphorylation Phosphorylation of p38 MAPK was measured by Western blotting with phospho-specific antibody reacting with phospho-p38 MAPK as described previously[14]. Briefly, cardiomyocytes were incubated in Hepes-buffer with low pH at room temperature for 1 h, then lysed with ice-cold lysis buffer. Lysate (50 μg of total protein) were separated by 12 % Tris-glycine/SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were probed with phospho-p38 MAPK antibody. The same membrane was stripped and then re-probed with a second primary antibody to determine the total protein abundance.

p38 MAPK activity assay p38 MAPK activity was detected as previously described[14]. Briefly, 200 μg of total protein was immunoprecipitated with immobilized phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibody. After wash out, the reaction was performed in kinase buffer containing 200 μmol/L ATP and 2 μg of ATF-2 fusion protein in the presence or absence of SB203580 (SB), a p38 MAPK inhibitor. The samples were boiled and subjected to SDS-PAGE gel. After the membrane was blocked, anti-phospho-ATF2 antibody was used to probe the activity of p38 MAPK.

Materials and antibodies Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. SB203580 was purchased from Calbiochem. Rabbit polyclonal antibodies against phospho-p38 MAPK and phospho-ATF2 were purchased from Cell Signaling Technology. Antibodies against total SAPK/p38 and anti-mouse IgG were purchased from Upstate.

Statistics All data were expressed as mean±SD. Student's *t*-test was used for simple comparisons and

ANOVA was used when appropriate. $P<0.05$ was considered to be statistically significant.

RESULTS

Isolation of cardiomyocytes Adult rat cardiomyocytes were isolated by a standard enzymatic technique. Under our experimental setting, about 90 % of freshly isolated cells were rod-shaped (Fig 1).

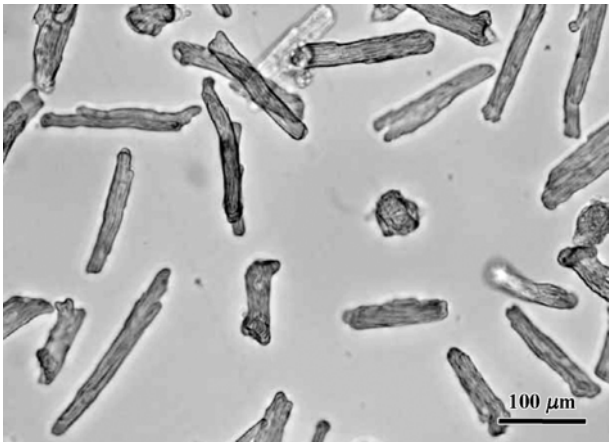

Fig 1. Adult rat cardiomyocytes immediately after isolation.

Effect of cardiomyocytes ischemia on p38 MAPK phosphorylation and pH_i Adult rat cardiomyocytes were isolated and pelleted to keep away from oxygen to create an *in vitro* ischemia model. Phosphorylation of p38 MAPK was measured to indicate the activation of p38 MAPK. Ischemia significantly increased p38 MAPK phosphorylation by about 2.7 fold, as shown by the typical Western blot example (Fig 2A, top band) and the average data (Fig 2B), with comparable total p38 MAPK protein level (Fig 2A, bottom band). As described previously, pH_i was always changed in response to ischemia. To examine the possible change of pH_i under our experimental conditions, we measured the effect of ischemia on pH_i, and found that ischemia treatment reduced pH_i significantly from 7.15±0.03 (control) to 6.56±0.13 (ischemia) (Fig 2C), while it markedly increased p38 MAPK activation.

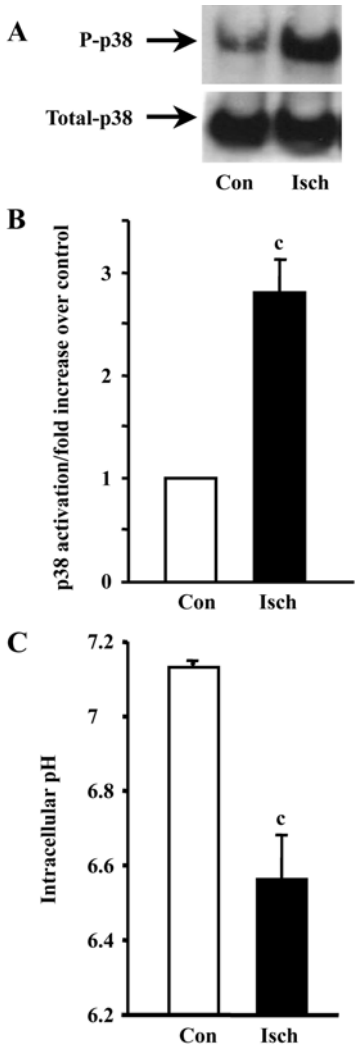

Fig 2. Effects of ischemia on p38 MAPK phosphorylation and intracellular pH in adult rat cardiomyocytes. Panel A (top band) shows increased p38 MAPK phosphorylation in response to ischemic treatment in cardiomyocytes. The same membrane was striped and reprobed with an antibody reacting with total p38 MAPK to show the kinase protein abundance (panel A, bottom band). Panel B shows the average data of ischemia-induced increase in p38 phosphorylation. *n*=3 independent experiments. Mean±SD. ($^cP<0.01$ *vs* control). Panel C illustrates ischemia-induced decrease in pH_i (*n*=30 cells from 3 heart for each group; $^cP<0.01$ *vs* control).

Response of p38 MAPK activation to low pH Since extracellular stresses are generally known to induce MAPKs, so next we examined the relationship between pH and p38 MAPK in adult rat cardiomyocytes. pH_i and pH_o are very closely linked, pH_o very probably affects cellular progress by changing pH_i, we first examined whether the pH_i is changed corresponding to treatments of pH_o and found that pH_i decreased gradually by low pH_o, from pH_i of 7.15±0.03 corresponding to pH_o of 7.4 to pH_i of 6.56±0.11 corresponding to pH_o of 5.5. Thus, we used Hepes-buffer with pH_o 5.5 (pH_i 6.56) to examine whether intracellular acidosis was an activator of p38 MAPK in adult rat cardiomyocytes. Phosphorylation of p38 MAPK was elevated following low pH treatment, reached the peak of about 3.1-fold augmentation (Fig 3A, B).

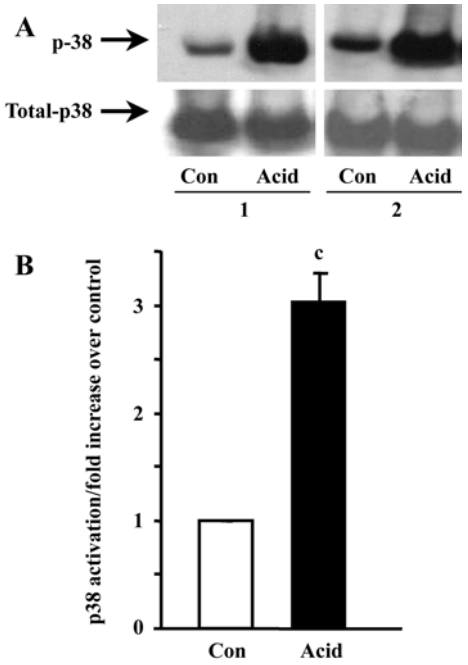

Fig 3. Responses of p38 MAPK phosphorylation to low extracellular pH (pH_o). Cardiomyocytes were incubated by the HEPES buffer with low pH (pH_i 6.56 induced by incubating cardiomyocytes with the HEPES buffer at pH of 5.5). Panel A were two representative examples showing that p38 MAPK phosphorylation was markedly increased in response to intracellular acidosis. Panel B showed the average data, p38 MAPK phosphorylation was elevated to about 3.1-fold (*n*=3, ^cP<0.01 *vs* control).

Effect of inhibition of p38 MAPK activation on acidosis-mediated negative inotropic effect It has been well established that acidosis suppresses cardiac myocyte contractility[18,19]. In light of our recent finding that enhanced p38 activation exhibited a robust negative inotropic effect *in vivo*[20] and in cultured cardiac myocytes[15], and the present result that acidosis was able to activate p38 MAPK, we hypothesized that p38 MAPK signaling was involved in intracellular acidosis-mediated inhibition of cardiomyocyte contractility. SB203580 (10 μmol/L), a specific inhibitor of p38 MAPK, completely inhibited acidosis-induced p38 MAPK activation, as evidenced by an abrogation of p38 MAPK-induced phosphorylation of ATF-2 (Fig 4). SB203580 at a threshold concentration (5 μmol/L) had no detectable effect on baseline contractility in cells with normal pH_o, whereas it exhibited a significant positive inotropic effect in cells-subjected to the acidic buffer (Fig 5A, 5B). Thus, the negative inotropic effect of acidosis is, at least in part, attributable to p38 MAPK activation. This reveals a previously unappreciated

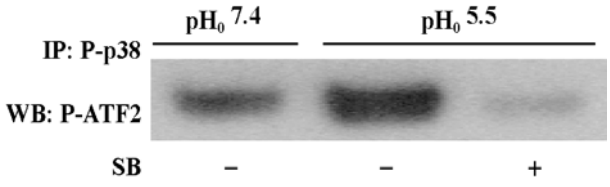

Fig 4. SB 203580 markedly inhibits p38 MAPK activation. p38 MAPK inhibitor, SB203580 (SB, 10 μmol/L), prevents p38 MAPK-mediated ATF-2 phosphorylation in response to an acidic buffer treatment.

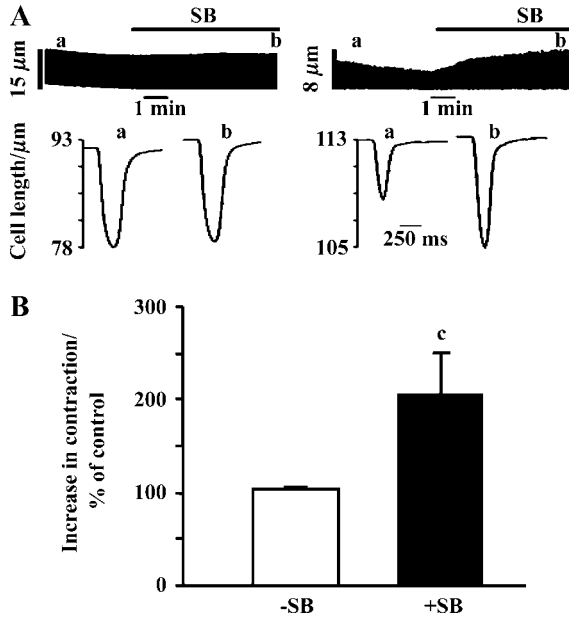

Fig 5. Inhibition of p38 MAPK significantly reversed acidosis-mediated negative inotropic effect in rat ventricular myocytes. Panel A showed the chart records of the contractile response to SB203580 (SB, 5 μmol/L) in representative myocytes perfused with the Hepes buffer with a normal pH 7.4 (left) or pH 6.5 (right). Panel B summarized the average contractile responses of cardiomyocytes in a low pH (pH 6.5) hepes buffer in the presence or absence of SB203580 (5 μmol/L). While SB203580 at this concentration had no effect on myocyte contraction amplitude, it significantly reversed acidosis-induced decrease in myocyte contractility (*n*=10-13 cells, ^cP<0.01 *vs* control).

mechanism underlying the mechanical failure of the ischemic injured heart.

DISCUSSION

A wide range of environmental stresses are known to induce activation of MAPKs and it has been reported that low pH can activate p38 MAPK homologue in yeast HOG1. In present study we investigated the p38 MAPK activation in response to low environment pH and its

potential role in regulating cardiac contractile response. Here we have some findings. Firstly, we demonstrated that in adult rat cardiomyocytes, intracellular pH was decreased during ischemic while the phosphorylation of p38 MAPK was increased. Secondly, we found that acidosis activated p38 MAPK. Finally, our data indicated that increased p38 MAPK activation might partially explain the inhibitory effect of acidosis on cardiac contraction.

Intracellular acidosis significantly activates p38 MAPK activation Myocardial ischemia and reperfusion was shown being accompanied by profound ionic alterations, including acidification, accumulation of sodium, and elevation of cytosolic calcium[21,22]. During the acute phase of ischemia, acidification occurs as a result of anaerobic metabolism, and preconditioning provides protective effect by attenuating the intracellular acidification during ischemia (or in isolated myocytes, during metabolic inhibition)[23,24]. There is general agreement that p38 MAPK is also activated by ischemia, although that the activation of this kinase is protective or deleterious to the ischemic heart is still a subject of controversy. We here reasoned that acidification might be related with p38 MAPK activation during ischemia. In our experimental model of isolated rat ventricular myocytes, ischemia concurrently induced a fall of pH_i by about 0.59 unit while activated p38 MAPK by about 2.7 fold (Fig 2). To explore whether the decrease of pH_i and the activation of p38 MAPK were parallel or linked with each other, we treated cardiac myocytes with buffer of pH 5.5 to mimic the intracellular acidosis condition. While the acid buffer with pH of 5.5 decreased pH_i to 6.5, it markedly increased the phosphorylation of p38 MAPK by about 3.1 fold (Fig 3). Recent study also reported that acidic conditions deregulated MAPK activation[25], however, it was under *in vitro* experimental conditions with synthesized enzymes of MAPKs. Thus, here these results first provide evidence in adult rat cardiomyocytes that acidification significantly activates p38 MAPK, and this may explain why both acidification and activation of p38 MAPK are always found during cardiac ischemia.

Acidosis-induced negative inotropic effect is partially mediated by p38 MAPK signaling Intracellular pH is an important regulator of cardiac contractility during receptor stimulation and under certain pathophysiological conditions. It is generally accepted that acidification inhibits cardiac myocyte contractility by reducing myofilament Ca^{2+} sensitivity[26]. Our recent studies have elucidated that activation of p38 MAPK decreases cell contractility, whereas inhibition of this kinase markedly increases cell contraction amplitude without altering sarcolemmal Ca^{2+} entry and intracellular Ca^{2+} transients, suggesting that p38 MAPK depresses the myofilament response to intracellular Ca^{2+} [15]. In the present study, we further demonstrated that the specific p38 MAPK inhibitor, SB203580, at the concentration (5 μmol/L) had no detectable effect on the contraction amplitude in cells with normal pH, but exhibited a marked positive inotropic effect in myocytes-subjected to the acidic conditions, indicating that p38 MAKP signaling may play an important role in acidosis-induced suppression of cardiomyocyte contractility. Based on these observations, it is reasonable to assume that p38 MAPK activation contributes, at least in part, to ischemia-associated dysfunction of myocardial contractility. This idea is substantiated by the fact that inhibition of p38 MAPK activity improves cardiac contractile function in ischemia/reperfusion-injured hearts or cardiac myocytes[27-30].

Physiological and pathophysiological relevance of low pH induced p38 MAPK activation The results of many studies demonstrate that ischemia is associated with intracellular acidification, and an acidification up to 1 pH unit has been reported[31]. The question is what are roles of p38 MAPK in cardiac ischemia. Although previous study reported that cytosolic alkalinization increased JNK and p38 MAPK activity[32], more recent studies demonstrated that p38 MAPK activity was required by NHE dependent cytoplasmic alkalinization[33,34]. In addition, in preconditioned hepatocytes, stimulation of p38 MAPK reduced acidosis[35]. Taking together with our present data, it is reasonable for us to hypothesize that cardiac ischemia induces acidosis and which consequently activates p38 MAPK, the activated p38 MAPK provides a negative feedback to the decreased pH_i by reducing acidosis through NHE dependent alkalinization. This will be tested in the near future.

At the cellular level acid conditions have dramatic effects on cell functions including cardiac contractility. It is known that contractile response is decreased during heart ischemia. Although the negative inotropic effect of acidosis is believed mainly at the level of myofilament responsiveness to Ca_i, it is possible that there remain other mechanisms. Our previous studies elucidated a novel role of p38 MAPK activation in regulation of cardiac contractility[14,15], this was further supported by our data presented here. The low pH mediated nega-

tive inotropic effect was partially reversed by perfusion with SB 203580 to inhibit p38 MAPK (Fig 5). Thus makes it reasonable to surmise that p38 MAPK activation mediated by acidosis during ischemia may, at least in part, contribute to ischemia induced decline of cardiac contractility.

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