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Stimulation of p38 MAP kinase reduces acidosis and Na^+ overload in preconditioned hepatocytes

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Abstract Ischemic preconditioning has been shown to improve liver resistance to hypoxia/reperfusion damage. A signal pathway involving A_{2A}-adenosine receptor, G_i-proteins, protein kinase C and p38 MAP kinase is responsible for the development of hypoxic preconditioning in hepatocytes. However, the coupling of this signal pathway with the mechanisms responsible for cytoprotection is still unknown. We have observed that stimulation of A_{2A}-adenosine receptors or of p38 MAPK by CGS21680 or anisomycin, respectively, appreciably reduced intracellular acidosis and Na^+ accumulation developing during hypoxia. These effects were reverted by p38 MAPK inhibitor SB203580 as well as by blocking vacuolar proton ATPase with bafilomycin A_1. SB203580 and bafilomycin A_1 also abolished the cytoprotective action exerted by both CGS21680 and anisomycin. We propose that the stimulation of p38 MAPK by preconditioning might increase hepatocyte resistance to hypoxia by activating proton extrusion through vacuolar proton ATPase, thus limiting Na^+ overload promoted by Na^+-dependent acid buffering systems.

Key words: Vacuolar proton ATPase; Liver preconditioning; Bafilomycin; Acidosis; Hypoxia; Liver injury; Liver transplantation

1. Introduction

Recent studies have demonstrated that resistance to prolonged ischemic injury can be acquired by the liver following one or more brief periods of ischemia followed by reperfusion [1–4]. This phenomenon, known as hepatic ischemic preconditioning, has received increasing interest since it increases the success of liver transplantation in rats [5,6]. The mechanisms leading to ischemic preconditioning have been extensively investigated in the heart, showing the involvement of the interstitial accumulation of endogenous mediators, and particularly of adenosine [7]. By interacting with myocardial adenosine A_1 receptors, adenosine triggers a signal pathway that involves heterotrimeric G_i-proteins, phospholipase C and the subsequent activation of protein kinase C (PKC) isoenzymes [7] as well as of a number of protein kinases including stress-activated protein kinases (SAPKs) also known as c-Jun N-terminal kinases (JNKs), extracellularly responsive kinases (ERKs) and p38 mitogen-activated protein kinase (p38 MAPK) [8–10]. Adenosine has also been implicated to play a role in liver preconditioning, however, differently from the heart, the stimulation of liver adenosine A_2 receptors is responsible for the hepatoprotective action [6,11].

Experiments performed using isolated rat hepatocyte suspensions demonstrated that the hepatoprotective action of liver preconditioning observed in vivo can be reproduced in vitro [4]. Using isolated hepatocytes we also observed that the development of hypoxic preconditioning involved the sequential activation of A_{2A}-adenosine receptor, G_i-proteins, phospholipase C and δ- and ε-PKC isoenzymes [12]. The stimulation of δ- and ε-PKCs was found to be coupled with the specific phosphorylation of p38 MAPK, whereas p38 MAPK inhibition reverted the effects of preconditioning [12]. Nonetheless, the coupling of p38 MAPK-mediated signals with the mechanisms preventing cell injury during hypoxia is still poorly defined.

Aim of this work has been to clarify how p38 MAPK stimulation by preconditioning contributes the development of hepatocyte protection against hypoxic injury. For this purpose, preconditioning was induced in isolated hepatocytes by the stimulation of A_{2A}-adenosine receptor with the specific A_{2A}-agonist CGS21680 as well as by the direct activation of p38 MAPK with anisomycin A.

2. Materials and methods

2.1. Materials

Collagenase (type I), *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), chelerythrine, nigericin, phenylmethylsulphonyl fluoride, 2*p*-(2-carboxyethyl)phetyl-amino-5′-*N*-ethylcarboxyamido-adenosine (CGS21680), anisomycin A, SB203580 and bafilomycin A_1 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium probe benzofuran isophthalate (SBFI)-acetoxymethyl ester, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) and propidium iodide were supplied by Molecular Probes (Eugene, OR, USA). All the other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2. Hepatocyte isolation and treatments

Isolated rat hepatocytes were prepared by collagenase liver perfusion [13] of fed male Wistar rats (180–250 g weight) (Nossan, Correzzana, Italy). The use and the care of the animals were approved by the Italian Ministry of Health and by the University Commission for Animal Care following the criteria of the Italian National Research Council. Cell viability, estimated at the beginning of the experiments, ranged between 85 and 90%. Hepatocytes were suspended in Krebs–Henseleit–HEPES (KKH) buffer (final cell density of 10^6/ml) containing 118 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.3 mmol/l $CaCl_2$, 25 mmol/l $NaHCO_3^-$ and 20 nmol/l HEPES at pH 7.4. Hepatocyte treatment with the different agents was performed

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by 15 min pre-incubation at 37°C before 90 min incubation at 37°C in sealed bottles under 95% N_2/5% CO_2 atmosphere. Cell viability was estimated by microscope counting the hepatocyte excluding trypan blue and by the determination of nuclear fluorescence staining with propidium iodide according to Gores et al. [14].

2.3. Measurement of lactate production

Hepatocyte lactate production was measured enzymatically according to Gutmann and Wahlefeld [15] in cell suspensions (3×10^6 cells/ml) incubated 30 min at 37°C under hypoxic and normoxic conditions and with or without the preconditioning agents CGS21680 and anisomycin A.

2.4. Measurement of cytosolic pH

Cytosolic pH was measured using the fluorescent indicator dye 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein-acetoxymethyl ester (BCECF-AM). Briefly, isolated hepatocytes (3×10^6 cells/ml) were loaded with BCECF-AM (5 μg/ml) by 30 min incubation at 25°C in a modified KHH buffer containing 2% bovine serum albumin and 10 mmol/l glucose. After washing, the cells were re-suspended in fresh KKH medium and treated with preconditioning agents. Preconditioned and non-preconditioned hepatocytes were then subjected to 20 min of hypoxia. At the end of the incubation, 2 ml aliquots were taken and centrifuged for 1 min at $800\times g$. Cell pellets were re-suspended in fresh medium and the fluorescence was determined at 450/500 nm wavelength pair using a Hitaki 4500 spectrofluorimeter. Calibration values were obtained for each experiment by incubating hepatocytes in media at different pH containing 120 mmol/l K^+ and 10 μmol/l of the K^+/H^+ ionophore nigericin.

2.5. Measurement of intracellular Na^+ content

For the measurement of intracellular Na^+ content isolated hepatocytes were loaded with the fluorescent Na^+ probe benzofuran isophthalate-acetoxymethyl ester (SBFI-AM; 10 μmol/l final concentration in DMSO) by 60 min incubation at 25°C in KHH buffer containing 2% bovine serum albumin and 10 mmol/l glucose. After washing, the cells were re-suspended in fresh KKH medium and preconditioned as described above. At each time point, 2 ml aliquots of the cell suspension were taken and centrifuged for 1 min at $800\times g$. Cell pellets were re-suspended in fresh medium and the fluorescence was determined at using Hitaki 4500 spectrofluorimeter set at 345 and 385 nm excitation and at 510 nm emission wavelengths. The ratio of fluorescence values obtained with 345 nm and 385 nm excitation were calculated after correction for spontaneous SBFI fluorescence. Calibration of SBFI fluorescence was performed by placing the hepatocytes into solutions of known Na^+ concentration in the presence of the Na^+ ionophore gramicidin D (2 μmol/l).

2.6. Data analysis and statistical calculations

The data were expressed as means ± standard deviation (S.D.). Statistical analysis was performed by Instat-3 statistical software (GraphPad Software Inc, San Diego, CA, USA) using one-way ANOVA test with Bonferroni's correction for multiple comparisons when more than two groups were analyzed. Distribution normality of all the groups was preliminary verified by Kolmogorov and Smirnov test. Significance was taken at 5% level.

3. Results and discussion

Previous studies have shown that the prevention of intracellular acidosis is a consistent feature in preconditioned tissues [7]. Table 1 demonstrates that a significant improvement of intracellular pH can be observed in hypoxic hepatocytes following the induction of preconditioning by the stimulation of adenosine A_2 receptors with CGS21680 (1 μmol/l). A similar effect was also obtained by promoting the activation of p38 MAPK with 500 ng/ml anisomycin A, whereas SB203580 (2 μmol/l), a selective inhibitor of p38 MAPK reverted the action of both CGS21680 and anisomycin A on hepatocyte pH (Table 1). The possibility that the activation of p38 MAPK might prevent hypoxic acidosis by modulating the acid production during hypoxia was excluded, since lactate accumulation was not different between control hepatocytes (13.1 ± 0.97 nmol/mg protein/min) and CGS21680- or anisomycin A-treated cells (12.6 ± 1.23 and 14.3 ± 1.71 nmol/mg protein/min, respectively). The activation of proton extrusion through Na^+/H^+ exchanger or vacuolar proton ATPase (V-ATPase) has been proposed to account for the attenuation of intracellular acidosis in preconditioned myocardiocytes [16,17]. In isolated hepatocytes, the block of Na^+/H^+ exchanger with 5-(*N*,*N*-dimethyl)-amiloride (DMA) (10 μmol/l) did not appreciably affect the attenuation of intracellular acidosis produced by CGS21680 and anisomycin A (Table 1). Conversely, the addition of bafilomycin A_1 (50 nmol/l), a specific inhibitor of V-ATPase [18], completely abolished the protection exerted by the above agents against hypoxic acidosis (Table 1).

V-ATPases are a family of widely distributed ATP-driven

Table 1
Changes in intracellular pH in hypoxic hepatocytes following the stimulation of p38 MAPK activity and effect of the modulation of Na^+/H^+ exchanger and V-ATPase

Treatments	T_0	20 min hypoxia
None	7.37 ± 0.020	7.04 ± 0.084[a]
CGS21680 1 μmol/l	7.37 ± 0.004	7.30 ± 0.018[b]
Anisomycin A 500 ng/ml	7.35 ± 0.130	7.29 ± 0.111[b]
SB203580 2 μmol/l	7.37 ± 0.011	7.01 ± 0.038[a]
CGS21680+SB203580	7.37 ± 0.088	7.06 ± 0.042[a,c]
Anisomycin A+SB203580	7.36 ± 0.106	7.09 ± 0.121[a,c]
5-(*N*,*N*-dimethyl)-amiloride 10 μmol/l	7.36 ± 0.082	6.99 ± 0.062[a]
CGS21680+5-(*N*,*N*-dimethyl)-amiloride	7.37 ± 0.118	7.29 ± 0.216[b]
Anisomycin A+5-(*N*,*N*-dimethyl)-amiloride	7.37 ± 0.078	7.27 ± 0.135[b]
Bafilomycin A_1 50 nmol/l	7.36 ± 0.065	7.02 ± 0.057[a,c]
CGS21680+bafilomycin A_1	7.36 ± 0.079	7.02 ± 0.118[a,c]
Anisomycin A+bafilomycin A_1	7.37 ± 0.108	7.09 ± 0.31[a,c]

Isolated hepatocytes suspended in KKH buffer were loaded with the fluorescent indicator dye 2′,7′-bis(carboxyethyl)-5,6-carboxy fluorescein-acetoxymethyl ester (BCECF-AM) (5 μg/ml) by 15 min incubation at 37°C. After washing, the cells were re-suspended in fresh KHH buffer and incubated 15 min with 1 μmol/l CGS21680 or 500 ng/ml anisomycin A. CGS21680- or anisomycin A-treated and untreated cells were then incubated 20 min under 95% N_2/5% CO_2 atmosphere with or without the different inhibitors. In order to block p38 MAPK, hepatocytes were pre-treated with SB203580 5 min before the addition of CGS21680 or anisomycin A. The results are means of three–five different experiments ± S.D.

[a]Statistical significance $P < 0.02$ versus hepatocytes before the hypoxic treatment.

[b]Statistical significance $P < 0.02$ versus control hepatocytes incubated under hypoxic conditions.

[c]Statistical significance $P < 0.02$ versus CGS21680- or anisomycin A-treated cells.

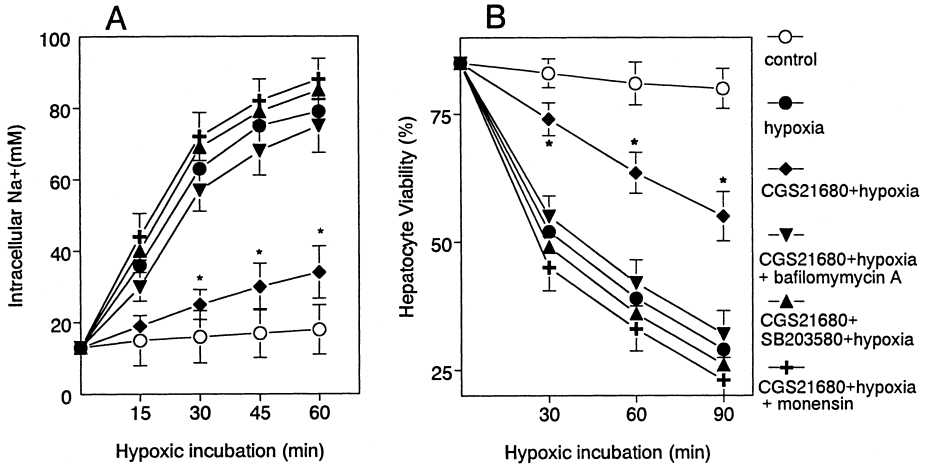

Fig. 1. Stimulation of adenosine A_2 receptors by CGS21680 reduces intracellular Na^+ accumulation (panel A) and cell death (panel B) in isolated hepatocytes exposed to hypoxia: effect of Na^+/H^+ ionophore monensin and of the inhibition of p38 MAPK and of V-ATPase with, respectively, SB203580 or bafilomycin A_1. Isolated rat hepatocytes suspended in KKH buffer were pre-incubated 5 min at 37°C with 2 μmol/l SB203580 or 15 min with 50 nmol/l bafilomycin A_1, before the addition of 1 μmol/l CGS21680. Monensin 10 μmol/l was added just before hypoxic incubation. Hepatocyte viability and intracellular Na^+ content were assessed by, respectively, propidium iodide and sodium probe benzofuran isophthalate (SBFI) fluorescence during 90 min incubation under 95% N_2/5% CO_2 atmosphere. The results are means of four–five different experiments. Statistical significance: $^*P < 0.001$ versus hepatocytes not receiving CGS21680 or incubated with CGS21680 in the presence of monensin, bafilomycin or SB203580.

proton pumps that are responsible for the acidification of intracellular compartments, such as synaptic vesicles, lysosomes and endosomes [19]. In several cell types, including phagocytes and hepatocytes, V-ATPases are present also in the plasma membranes, where they contribute in controlling intracellular pH [20,21]. The mechanisms regulating V-ATPase activity have been extensively investigated showing the involvement of reversible subunits assembly and disulfide bond formation [22]. Nonetheless, Nordström and co-workers have reported that PKC-mediated signaling can also be responsible for plasma membrane V-ATPase regulation during rat phagocyte metabolic burst [23]. Taken together these results suggested that the stimulation of p38 MAPK in preconditioned hepatocytes improved cellular pH during hypoxia by favoring proton extrusion through vacuolar proton ATPase.

One of the consequences of hypoxic acidosis is an increased Na^+ influx due to the activation of acid buffering systems involving Na^+/H^+ exchanger and Na^+/HCO_3^- co-transporter [24]. Such an effect, in combination with the impairment of Na^+ efflux through Na^+/K^+ ATPase due to ATP depletion, leads to a progressive accumulation of Na^+ within the cells. The measurement of intracellular Na^+ levels during hypoxic incubation demonstrated a significant reduction of Na^+ accumulation in hepatocytes receiving CGS21680 (Fig. 1) or anisomycin A (Fig. 2). The effects of CGS21680 and of anisomycin A on intracellular Na^+ were not affected by blocking

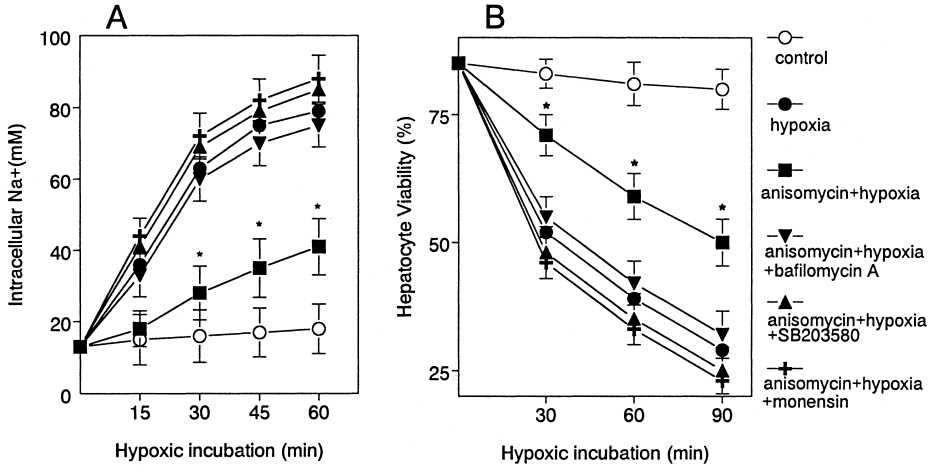

Fig. 2. Activation of p38 MAPK by anisomycin A reduces intracellular Na^+ accumulation (panel A) and cell death (panel B) in isolated hepatocytes exposed to hypoxia: effect of Na^+/H^+ ionophore monensin and of the inhibition of p38 MAPK and of V-ATPase with, respectively, SB203580 or bafilomycin A_1. Isolated rat hepatocytes suspended in KKH buffer were pre-incubated 5 min at 37°C with 2 μmol/l SB203580 or 15 min with 50 nmol/l bafilomycin A_1, before the addition of or 500 ng/ml anisomycin A. Monensin 10 μmol/l was added just before hypoxic incubation. Hepatocyte viability and intracellular Na^+ content were assessed by, respectively, propidium iodide and sodium probe benzofuran isophthalate (SBFI) fluorescence during 90 min incubation under 95% N_2/5% CO_2 atmosphere. The results are means of four–five different experiments. Statistical significance: $^*P < 0.001$ versus hepatocytes not receiving anisomycin A or incubated with anisomycin A in the presence of monensin, bafilomycin or SB203580.

Na^+/K^+ ATPase with ouabain (not shown). Conversely, inhibiting p38 MAPK with SB203580 or blocking V-ATPase with bafilomycin A_1 reverted the protection against Na^+ accumulation induced by either CGS21680 (Fig. 1) or anisomycin A (Fig. 2). This suggests that, by preventing intracellular acidosis, the activation of p38 MAPK might limit Na^+ influx through Na^+-dependent acid buffering systems. Recent studies have indicated that the activation of Na^+-K^+-Cl^- co-transporter and of non-selective cation channels are also involved in promoting Na^+ influx within liver cell exposed to hypoxia or to oxidative stress [25,26]. At this stage it can not be excluded that p38 MAPK-mediated signals might also regulate these latter Na^+ transport mechanisms in hepatocyte plasma membranes.

We have previously reported that the derangement of cellular osmotic equilibrium consequent to Na^+ overload preceded hepatocyte killing by metabolic inhibition [13,27]. Furthermore, conditions that prevented Na^+ overload were found protective against the cytotoxic effects of hypoxia, mitochondrial damage or reperfusion injury [13,28,29]. Figs. 1 and 2 show that SB203580 and bafilomycin A_1 reverted the protection given by CGS21680 or anisomycin A against hepatocyte killing during hypoxia. A similar effect was also seen following the addition of Na^+ ionophore monensin (Figs. 1 and 2). Nonetheless, monensin, SB203580 and bafilomycin A_1 did not affect Na^+ content and cell viability when added to hepatocytes maintained under normoxic conditions (not shown).

In conclusion, we propose that the stimulation of p38 MAPK by preconditioning might increase hepatocyte resistance to hypoxia by activating proton extrusion through vacuolar proton ATPase, limiting Na^+ influx promoted by Na^+-dependent acid buffering systems. Recently, inhibition of caspase 3 has been reported to prevent hepatocyte and sinusoidal endothelial cell apoptosis in preconditioned mouse livers [3]. This latter observation is compatible with the present findings, since modulation of V-ATPase can stimulate apoptosis by enhancing p53 expression and caspase 3 activity [30].

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