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Changes in Mitogen-activated Protein Kinase Activities During Acidification-induced Apoptosis in CHO Cells

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Homeostatic pH is very important for various cellular processes, including metabolism, survival, and death. An imbalanced-pH might induce cellular acidosis, which is involved in many abnormal events such as apoptosis and malignancy. One of several factors contributing to the onset of metabolic acidosis is the production of lactate and protons by lactate dehydrogenase (LDH) in anaerobic glycolysis. LDH is an important enzyme that catalyzes the reversible conversion of pyruvate to lactate. This study sought to examine whether decreases in extracellular pH induce apoptosis of CHO cells, and to elucidate the role of mitogen-activated protein kinases (MAPKs) in acidification-induced apoptosis. To test apoptotic signaling by acidification we used CHO *dhfr*$^-$ cells that were sensitive to acidification, and CHO/anti-LDH cells that are resistant to acidification-induced apoptosis and have reduced LDH activity by stable LDH antisense mRNA expression. In the present study, cellular lactic acid-induced acidification and the role of MAPKs signaling in acidification-induced apoptosis were investigated. Acidification, which is caused by HCO_3^--free conditions, induced apoptosis and MAPKs (ERK, JNK, and p38) activation. However, MAPKs were slightly activated in acidic conditions in the CHO/anti-LDH cells, indicating that lactic acid-induced acidification induces activation of MAPKs. Treatment with a p38 inhibitor, PD169316, increased acidification-induced apoptosis but apoptosis was not affected by inhibitors for ERK (U0126) or JNK (SP600125). Thus, these data support the hypothesis that activation of the p38 MAPK during acidification-induced apoptosis contributes to cell survival.

Keywords: CHO (Chinese hamster ovary) cells, acidification, apoptosis, MAPKs

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Introduction

Properly regulated pH is critical for cellular functions, including metabolism, calcium homeostasis, gene expression, motility, and adhesion (Puceat, 1999; Nagy *et al.*, 2000; Terminella *et al.*, 2002; Boron, 2004). Therefore, aberrant regulation of pH may be involved in numerous aspects of negative cellular activity such as apoptosis, malignancy, and contractility (Furlong *et al.*, 1997; Liu *et al.*, 2000; Hirpara *et al.*, 2001). It is further implicated in the pathogenesis of particular forms of renal stones (Dent's disease) (Capuano and Capasso, 2003). Acidic conditions, which are caused by a pH imbalance, also have varying effects on cellular functions, including cell survival and cell death (Kreisberg, 1980; Siesjo *et al.*, 1996; Zheng *et al.*, 2004). To maintain normal cellular metabolism, it is essential that pH is maintained within a narrow range (Wadsworth and van Rossum, 1994; Halestrap and Price, 1999; Juel and Halestrap, 1999; Zhou, 2001). One of several mechanisms for metabolic acidosis is the production of lactic acid by lactate dehydrogenase (LDH) in the anaerobic glycolytic pathway (Jeong *et al.*, 2001).

LDH is an important enzyme catalyzing the reversible transformation of pyruvate to lactate (Jeong *et al.*, 2001; Koukourakis *et al.*, 2003). LDH is a tetrameric protein with five isotypes (A_4, A_3B, A_2B_2, AB_3 and B_4) composed of two subunits (LDH-A and LDH-B). LDH-A catalyzes the

conversion of pyruvate to lactate in anaerobic glycolysis (Jeong *et al.*, 2001). Recently, it has been reported that the increased susceptibility to stress-induced apoptosis associated with Myc transfection has been linked to the overexpression of the LDH-A gene (Shim *et al.*, 1998; Papas *et al.*, 1999). The LDH-B subunit primarily converts lactate to pyruvate in aerobic tissue, such as heart tissue. Lactic acid has a pK_a of 3.86, and is dissociated at physiological pH as lactate anions and protons (Jeong *et al.*, 2001). Thus, up-regulation of LDH ensures an efficient glycolytic metabolism under anaerobic conditions and leads to acidification-induced apoptosis (Jeong *et al.*, 2001; Tiefenthaler *et al.*, 2001).

In the present study, Chinese hamster ovary (CHO) cells and CHO/anti-LDH cells with reduced LDH activity by stable LDH-A antisense mRNA expression were used to study the effect of pH by changing the intracellular lactate contents. The engineered CHO cells are resistant to cytosolic acidification (Jeong *et al.*, 2001). It has been reported that mitogen-activated protein kinase (MAPKs) pathways of mammalian cells are stimulated by lower environmental pH (Feifel *et al.*, 2002; Zheng *et al.*, 2004). MAPKs are important intermediates in signal transduction pathways that affect cell growth, differentiation, apoptosis, and cellular responses to stress (Davis, 1993; Chang and Karin, 2001; Yoshizumi *et al.*, 2003; Zhou *et al.*, 2004). However, the regulation of MAPKs in acidification-induced apoptosis is still unknown. This study sought to examine whether decreases in extracellular pH by incubation of CHO cells in HCO_3^--free conditions induced cellular apoptosis of the cells, and also to elucidate the role of MAPKs in acidification-induced apoptosis. The findings herein suggest that the activation of p38 MAPK contributes to cell survival during cellular acidification.

Materials and Methods

Cells and cell culture

CHO/*dhfr*$^-$ cells and CHO/anti-LDH cells were maintained in F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 14 mM sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin at 37°C in a humidified HCO_3^--supplied incubator. To examine the effects of pH alteration on apoptosis, cells were grown in 10 mM HEPES-buffered F-12 medium (pH 7.5 and pH 6.5) in a HCO_3^--free incubator.

Preparation of MAPK inhibitors

MAPK inhibitors (U0126, SP600125, and PD169316; EMD Biosciences, Darmstadt, Germany) were dissolved in dimethyl sulfoxide (DMSO). The cell cultures were treated with these inhibitors at a final concentration of 10 μM in the HEPES-buffered F-12 media.

Flow cytometry analysis

After incubating the cells in HCO_3^--supplied or -free incubators for the indicated times, whole cells (floating and adherent cells) were prepared for flow cytometric analysis as described in Asada *et al.* (1999) with some modifications. Cells were washed with phosphate-buffered saline (PBS), trypsinized, and centrifuged. The cell pellets were resuspended in PBS containing 5 mM EDTA and permeabilized by incubation in 70% ethanol at -20°C for more than 24 h. After centrifugation, the cell pellets were suspended in PBS containing 5 mM EDTA, treated with RNase A (50 μg/ml) at room temperature for 30 min, and stained with 50 μM propidium iodide (Sigma, Saint Louis, MO) for 10 min in the dark. Stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and data are expressed as the percentages of apoptotic cells relative to the total number of cells.

Western blot analysis

After cells were incubated in a HCO_3^--free incubator for the indicated time points, cells were washed with ice-cold PBS and lysed with a lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, and protease inhibitor cocktail). Lysates were clarified by centrifugation at 12,000×g for 10 min at 4°C. Cytosolic extracts were denatured using SDS sample buffer (200 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 0.4% bromophenol blue, and 400 mM β-mercaptoethanol) and separated by gel electrophoresis on a 10% SDS-polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane, and the levels of MAPKs were detected using MAPK-specific antibodies and the ECL reagent (Cell Signaling Technology, Beverly, MA).

Statistical analysis

All values were expressed as the means of three experiments±S.D. Statistical significance was evaluated by ANOVA, using the STATISTICA 6.0 software package. If treatments were determined to be significant, pairwise comparisons were performed using Scheffe's adjustment. Differences were considered statistically significant for p values <0.05.

Results

Cellular acidification and apoptotic events under a HCO_3^--free condition

Many studies have shown that the lactate anions and protons, which are produced during anaerobic glycolysis, decrease intracellular and extracellular pH. This reduction in cellular pH leads to acidification-induced apoptosis (Tiefenthaler *et al.*, 2001). To demonstrate the acidification induced-apoptosis, CHO cells were cultured in sodium bicarbonate-buffered F-12 medium in a humidified HCO_3^--

supplied incubator for 24 h, and then grown in HEPES-buffered F-12 medium (pH 7.5) in a HCO_3^--free incubator. The apoptotic nature of cell death was morphologically confirmed via phase-contrast microscopy, showing that the CHO cells exhibited membrane blebbing and the loss of their attachment to the dish, as compared with the antisense LDH-A RNA expressing cells (Fig. 1). Additionally, apoptosis was determined by measuring the DNA content of cells after staining with propidium iodide (Jeong *et al.*, 2001). The pH in the media was decreased and the percentage of apoptotic cells gradually increased after exposure to a HCO_3^--free incubator (Fig. 2).

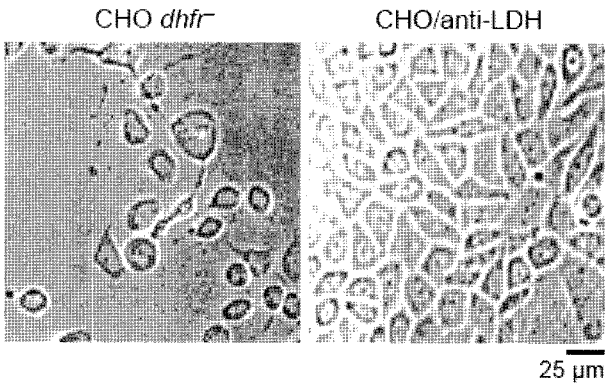

Fig. 1. Morphological changes due to acidification. Cells were incubated in a HCO_3^--free incubator for 48 h and photographed using a light microscope.

MAPKs activation response in acidic conditions

MAPK signaling cascades are multifunctional signaling networks that regulate cell growth, differentiation, and apoptosis (Davis, 1993). To examine the role of MAPKs during acidification-induced apoptosis, activation of MAPKs was analyzed by Western blot. After culturing the cells in a HCO_3^--supplied incubator for 24 h, cells were transferred to a HCO_3^--free incubator for each time point. Three MAPKs' (extracellular signal-regulated protein kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38) activities were elevated during HCO_3^--free conditions (Fig. 3A). To compare the activation of MAPKs between CHO and CHO/anti-LDH cells, these cells were incubated in a HCO_3^--supplied and -free condition for 1 h. Whereas MAPKs were activated in CHO cells, MAPKs in CHO/anti-LDH cells were only

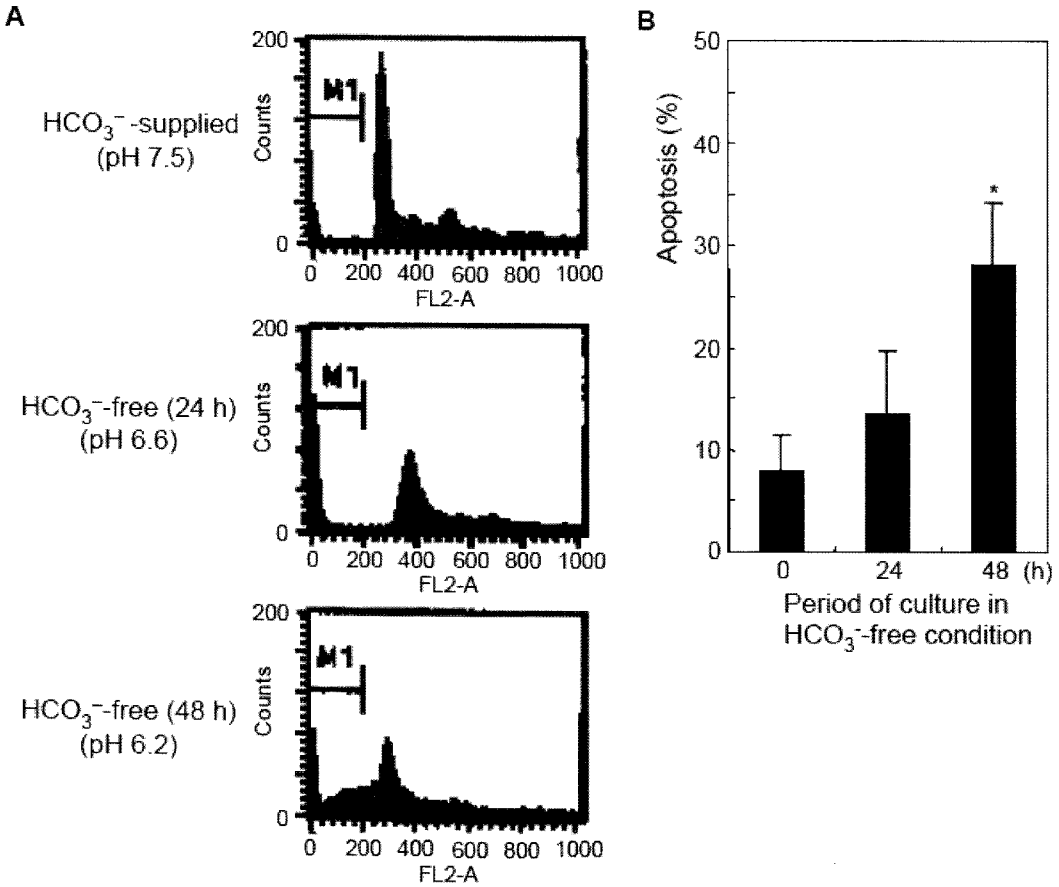

Fig. 2. Apoptosis of cells in a HCO_3^--free condition. Cells (2 x 10^5/60-mm dish) were cultured in sodium bicarbonate-buffered F-12 medium under a HCO_3^--supplied incubator for 24 h, and then incubated in HEPES-buffered F-12 medium (pH 7.5) in a HCO_3^--free incubator. Apoptosis was determined by measuring the DNA content of cells after staining with propidium iodide. Data are expressed as percentages of apoptotic cells relative to the total number of cells (n = 3). *, $p < 0.05$ *versus* HCO_3^--supplied control cells.

marginally activated in the HCO_3^--free incubator (Fig. 3B). These findings suggest that the extent of LDH expression can regulate cellular acidification and activities of MAPKs during this period.

Effect of MAPK inhibitors on acidification-induced apoptosis

We observed that MAPKs were activated in wild-type CHO cells under acidic conditions. To examine the role of the activated MAPKs in acidification, CHO cells were treated with MAPK inhibitors. Cells that were treated with ERK and JNK MAPK inhibitors and then incubated in a HCO_3^--free incubator for 24 h, did not show any differences in the regulation of acidification-induced apoptosis. However, cells that were treated with p38 inhibitor underwent increased apoptosis compared with the untreated control cells (Fig. 4). These results suggest that the activation of p38 may be implicated in cell survival and resistance to acidification-induced apoptosis.

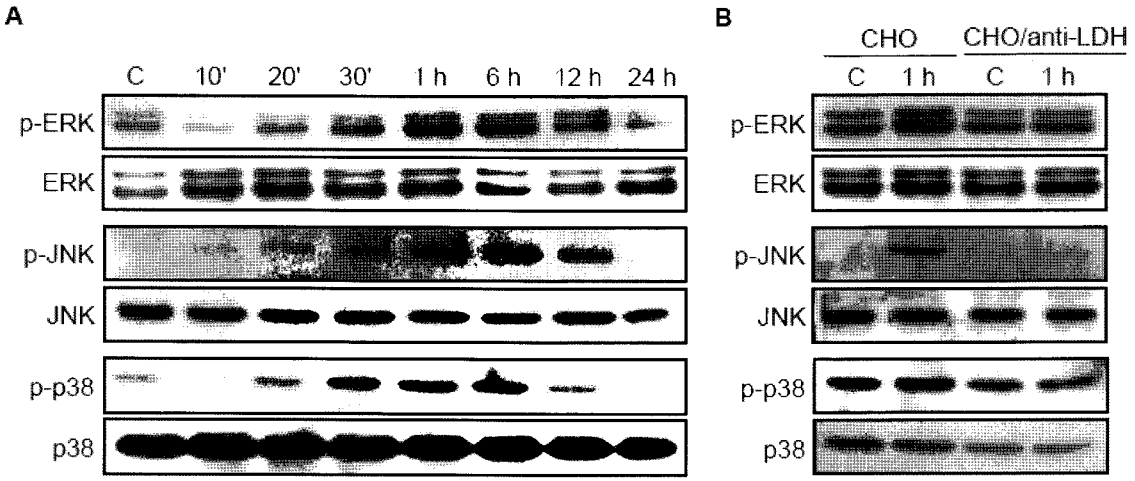

Fig. 3. Activation of MAPKs in a HCO_3^--free condition. After culturing the cells in a HCO_3^--supplied incubator, cells were incubated with HEPES-buffered F-12 medium (adjusted to pH 6.5) for the indicated times in a HCO_3^--free incubator. (**A**) MAPKs (ERK, JNK, and p38) in CHO cells were activated under acidic conditions. (**B**) MAPKs in CHO/anti-LDH cells were slightly activated under a HCO_3^--free condition. ERK, extracellular signal-regulated kinase; p-ERK, phospho-ERK; JNK, c-Jun NH_2-terminal kinase; p-JNK, phospho-JNK; p-p38, phospho-p38.

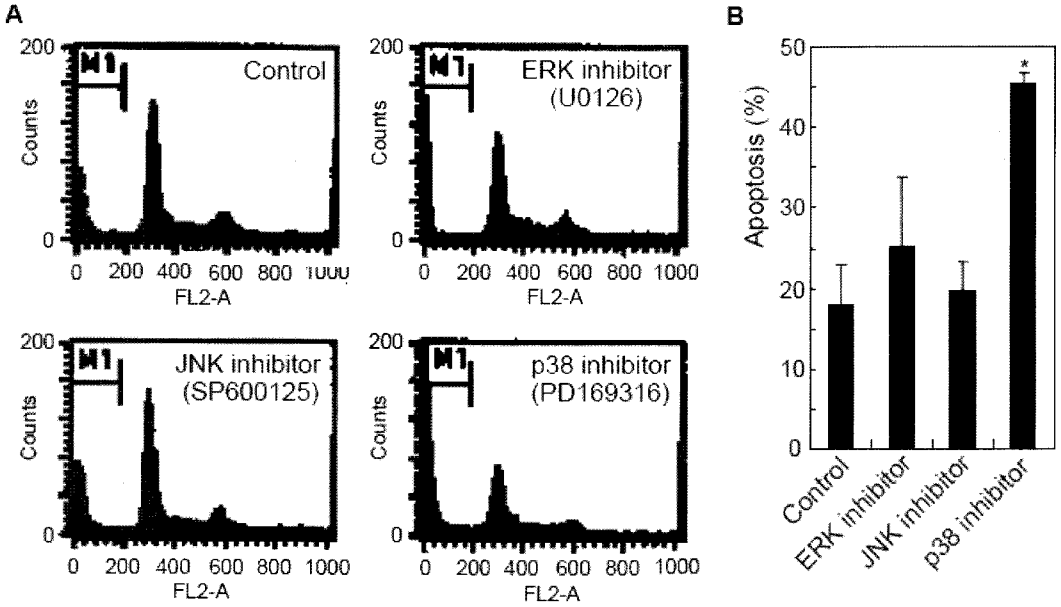

Fig. 4. Effect of MAPK inhibitors on acidification-induced apoptosis. Cells (5×10^5/60-mm dish) were treated with MAPK inhibitors (ERK, JNK, and p38 inhibitors, 10 μM final concentration) in a HCO_3^--free incubator for 24 h, stained with propidium iodide, and then analyzed by flow cytometry. Data are expressed as percentages of apoptotic cells relative to the total number of cells (n=3). *, $p<0.05$ *versus* vehicle-treated control.

Discussion

It has been reported that MAPKs influence cell growth, differentiation, apoptosis, and cellular responses to stress (Davis, 1993; Chang and Karin, 2001; Yoshizumi *et al.*, 2003; Zhou *et al.*, 2004). Several studies have reported a correlation between intracellular acidification and the onset of apoptosis (Reynolds *et al.*, 1996; Furlong *et al.*, 1997; Shrode *et al.*, 1997). Thus, acidification is a possible signal mediator during apoptosis. It has also been reported that sequential apoptotic reactions by lactic acid-induced acidification in CHO cells cause reduction of the mitochondrial membrane potential, release of cytochrome C, activation of caspase-3, and DNA fragmentation (Jeong *et al.*, 2001). However, the regulation of MAPKs in acidification-induced apoptosis of CHO cells is still entirely unclear. In the present study, the role of MAPKs in acidification-induced apoptosis was examined.

The pH in the cellular cytosol must be maintained within a narrow range around pH 7.4. The maintenance of a stable physiological pH is of critical importance to mammalian survival. Disturbances in pH tend to contribute to several disorders, including chronic diarrhea, renal failure, and osteoporosis. Several studies reported that MAPK signaling might be implicated in these disorders. In mammals, there are four major groups of MAPKs: ERK1/2, JNK1/2/3, p38α/β/γ/δ (p38 family of kinases), and ERK5. The ERKs are generally responsive to growth factors and contribute to cellular proliferation, development, differentiation, and survival. Both the JNKs and the p38s are activated in response to cytokines and stress. The activation of JNKs contributes to apoptosis, inflammation, and tumorigenesis. The active forms of p38 affect cell motility, apoptosis, chromatin remodeling, and osmoregulation (Yoshizumi *et al.*, 2003; Zhou *et al.*, 2004). It is also reported that p38 is activated in low extracellular pH (HEPES-buffered system at pH 5.5) (Zheng *et al.*, 2004).

In vitro acidification can be induced using two buffering systems. First, metabolic acidosis is induced because of a reduced bicarbonate concentration. Generally, this acidosis is created by using a HEPES-buffered culture media (Jeong *et al.*, 2001). Second, respiratory acidosis is produced by an increased partial pressure of carbon dioxide (Ladoux *et al.*, 1988). In the present study, the pH of HEPES-buffered F-12 medium is constantly maintained under HCO_3^--free conditions, but altered in bicarbonate-buffered medium under HCO_3^--supplied conditions. This suggests that the HEPES-buffered system is suitable for the identification of MAPK signaling by acidification. We showed here that extracellular acidification, which is caused by pumping protons from the cell in the HEPES-buffered culture media, gradually induced apoptosis in CHO cells. All of the MAPKs (ERK, JNK, and p38) were activated during the acidosis of CHO cells. In comparison, MAPKs were only marginally activated in CHO/anti-LDH cells. These results suggest that MAPKs are activated by lactic acid-induced acidification and might be involved in the regulation of cell survival and death during lactic acid-induced acidification.

To demonstrate the role of MAPKs activation in acidification-induced apoptosis, MAPK inhibitors (U0126, SP600125, and PD169316) were used. The response of cells treated with ERK and JNK inhibitors was similar to the response of the control cells. However, treatment with the p38 inhibitor promoted apoptosis more than in the control cells. Thus, understanding p38 might be helpful in the study of various pathogeneses, which are caused by lactic acid-induced acidification. However, further study will be required to investigate the activation mechanisms of MAPKs in acidification-induced apoptosis. Our results indicate that the activation of p38 offers a protective effect in acidification-induced apoptosis. Thus, activation of p38, at least in part, contributes to cell survival in acidic conditions.

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