Extracellular alkalosis activates ERK mitogen-activated protein kinase of vascular smooth muscle cells through NADPH-mediated formation of reactive oxygen species

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Abstract Extracellular alkalosis induced phosphorylation of extracellular signal-regulated kinase (ERK) and enhanced serum-induced ERK phosphorylation in cultured rat aortic smooth muscle cells. While extracellular alkalinization increased verapamil-sensitive $^{45}Ca^{2+}$ uptake into the cells, ERK phosphorylation induced by extracellular alkalosis was not affected by verapamil. On the other hand, probes for oxidant signaling, such as superoxide dismutase, 4,5-dihydroxy-1,3-benzene-disulfonic acid, a cell-permeable antioxidant, and diphenyliodonium, a NADPH oxidase inhibitor, inhibited extracellular alkalosis-induced phosphorylation of ERK. These results suggest that activation of ERK induced by extracellular alkalosis is not dependent on transplasmalemmal Ca^{2+} entry but is caused by reactive oxygen species derived from an activation of NADPH oxidase.

Key words: Alkalosis; Mitogen-activated protein kinase; Ca^{2+} channel; Vascular smooth muscle; NADPH oxidase; Oxygen stress

1. Introduction

Extracellular signal-regulated kinase (ERK) is a key enzyme that regulates proliferation of vascular smooth muscle cells (VSMC) during the progress of atherosclerosis [1]. ERK has also been shown to be involved in regulation of vascular smooth muscle contractility [2]. Changes in extracellular pH are known to affect contractility of VSMC: an increase or a decrease in extracellular proton concentration attenuates and augments vasocontractility, respectively [3–5]. As a mechanism of these effects of extracellular pH changes, it is known that the concentration of extracellular proton affects Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCC) [6,7]. Changes in extracellular pH also influence intracellular pH [8–10], and intracellular acidification has been shown to activate ERK in the renal proximal tubule [11]. However, the relationship of extracellular pH with activation of ERK in VSMC has not been clarified. Moreover, it is still not clear whether activity of ERK is dependent on extra- and intracellular Ca^{2+} concentrations of VSMC.

NADPH oxidases are a group of plasma membrane-associated enzymes found in a variety of cells of mesodermal origin [12]. NADPH oxidase is also expressed in VSMC and thought to be a major source of superoxide [13], which has recently been shown to be involved in upward regulation of ERK phosphorylation by angiotensin II or mechanical stretch [14,15]. A recent study has also shown that H^+ channels optimize NADPH oxidase function by inhibiting membrane potential in polynuclear leukocytes [16], suggesting a relationship between extra- and intracellular pH and ERK activity. In the present study, using rat aortic smooth muscle cells, we therefore investigated the effects of extra- and intracellular pH changes on activity of ERK and whether pH effects on ERK activation are dependent on Ca^{2+} influx or NADPH oxidase activation.

2. Materials and methods

2.1. Preparation of vascular smooth muscle cells

Strains of VSMC were established from rat thoracic aortae (12-week-old male Wistar rats) by the modified explant method as described previously [17]. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). At confluence, cells displayed a 'hill and valley' growth pattern. All VSMC cultures used in this study were between the 10th and 25th passages. After the cells had reached the subconfluent stage, they were incubated with serum-free medium for 48 h. At 1 h prior to various treatments, the medium was replaced with DMEM containing 20 mM HEPES (pH 7.4). In some experiments, the cells were pretreated with verapamil, 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron), superoxide dismutase (SOD), diphenyliodonium (DPI) or a vehicle for 30 min before changing extracellular pH. Extracellular alkalosis or acidosis was induced by addition of Tris or HEPES to elevate or lower the pH of the medium from 7.4 to 7.9 or 6.9, respectively. An adequate volume of Tris or HEPES to change the pH as above was determined in preliminary experiments.

2.2. Western blotting analysis

Western blot analysis was performed as described previously [18]. After various treatments, the cells were washed with cold phosphate-buffered saline (PBS), and lysed by cold lysis buffer [mM: Tris–HCl (pH 7.4) 50, NaF 1, NaCl 150, EGTA 1, Na_3VO_4 1 and phenylmethylsulfonyl fluoride 1], containing 0.25% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 μg/ml aprotinin and 1 μg/ml leupeptin, on the ice. Then, the cells in each well were scraped off, transferred to an Eppendorf tube, and sonicated for 5 s. The protein supernatant was separated by centrifugation, and protein concentration was determined with the Bio-Rad protein assay reagent (Bio-Rad

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DPI, diphenyliodonium; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; VDCC, voltage-dependent Ca^{2+} channels; VSMC, vascular smooth muscle cells

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Laboratories). An equal amount of each protein (5 μg) was loaded on a 10% SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was blocked with a blocking buffer containing Tris-buffered saline and 0.1% Tween 20 with 5% w/v non-fat dry milk and incubated for 1 h at room temperature. The membrane was then incubated with rabbit anti-phosphospecific ERK-1/ERK-2 antibody (Cell Signaling) and rabbit anti-ERK-1/ERK-2 antibody (Sigma) diluted 1:1000 overnight at 4°C. Next, detection was performed by alkaline phosphatase-conjugated anti-rabbit secondary antibodies and enhanced chemiluminescence (Bio-Rad Laboratories). Densitometric analysis was used to quantify each protein level.

2.3. Measurement of $^{45}Ca^{2+}$ uptake

Measurement of $^{45}Ca^{2+}$ uptake was performed as described previously [19]. After serum starvation for 48 h, the cells were washed once with PBS containing 0.1 mM EGTA. The medium was then replaced with DMEM containing 5 mM HEPES/Tris and 0.1 mM $CaCl_2$ (pH 7.4) for 1 h and next exposed to various conditions with 0.4 μCi/ml of $^{45}CaCl_2$. After 20 min incubation, the cells were washed with ice-cold Ca^{2+}-free PBS containing 0.1 mM EGTA and solubilized with 2 N NaOH. The radioactivity of the solubilized cell suspension was detected with a liquid scintillation counter.

2.4. Data analysis

At least three separate experiments of Western blotting were performed for comparison of expression of phosphorylated signal proteins. The data are expressed as mean values with S.D. Statistical analysis was done using analysis of variance followed by Scheffé's *F*-test.

3. Results and discussion

Phosphorylated ERK was only slightly detected in the cells incubated in serum-free media with pH 6.9 and pH 7.4 for 20 min (Fig. 1A). Elevation of extracellular pH from 7.4 to 7.9 caused significant expression of both phosphorylated ERK1 and ERK2. Addition of FBS to medium with pH 7.4 also resulted in activation of ERK. This serum-induced activation of ERK was significantly higher and lower in media with pH 7.9 and with pH 6.9, respectively, than in the control medium

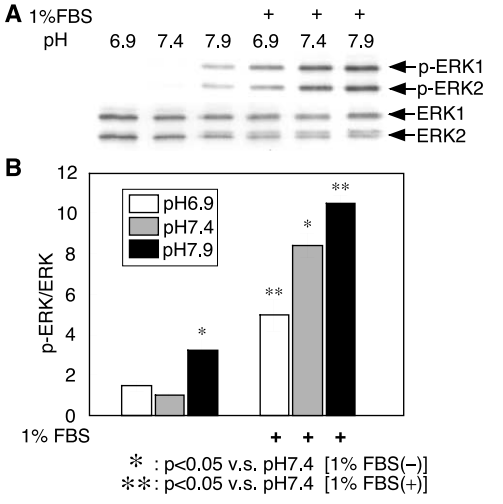

Fig. 1. A: Effects of extracellular pH on ERK phosphorylation in the absence or presence of FBS in rat aortic smooth muscle cells. Cells were stimulated in media with different values of extracellular pH for 20 min with or without 1% FBS. B: Quantitative immunoblot analysis of ERK phosphorylation. Phosphorylated ERK was normalized to the total form and then expressed as relative fold of the phosphorylation level in the condition with extracellular pH of 7.4. Means ± S.D. of three independent experiments are shown.

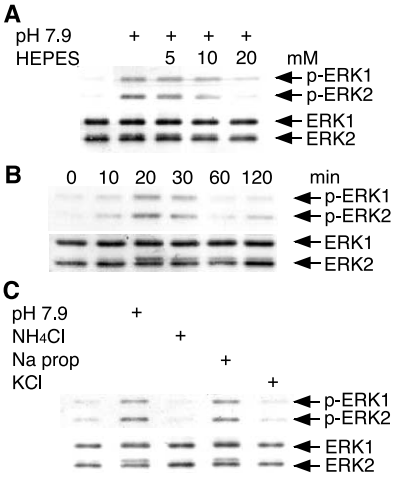

Fig. 2. A: Effects of addition of HEPES on extracellular alkalosis-induced activation of ERK. Different concentrations of HEPES were added to the medium simultaneously with Tris, which elevates the pH of the medium from 7.4 to 7.9. Similar results were obtained from at least three separate experiments. B: Time course of ERK phosphorylation induced by extracellular alkalosis. Similar results were obtained from at least three separate experiments: C. Effects of intracellular alkalinization and acidification induced by NH_4Cl (10 mM) and sodium propionate (Na prop, 20 mM), respectively, on ERK phosphorylation. Equivalence of alkalotic and acidic power resulting from 10 mM NH_4Cl and 20 mM propionate, respectively, was confirmed by intracellular pH measurement in a preliminary study. Effects of KCl (60 mM) on ERK phosphorylation were also shown. Similar results were obtained from at least three separate experiments.

with pH 7.4 (Fig. 1A,B). Neutralization of Tris-induced extracellular alkalosis by addition of HEPES abolished the induction of phosphorylated ERK (Fig. 2A). Thus, extracellular alkalosis was demonstrated to be one of the stimuli that induce activation of ERK. Extracellular alkalosis-induced activation of ERK reached a peak at 20 min after changing pH and then declined (Fig. 2B). On the other hand, p38 mitogen-activated protein kinase was not activated by Tris-induced extracellular alkalosis in VSMC (data not shown). Since extracellular pH also affects intracellular pH, we also carried out an experiment to determine whether ERK is affected by changes in intracellular pH. Extracellular applications of NH_4Cl and propionate are well-known maneuvers for inducing intracellular alkalinization and acidification, respectively, without changing extracellular pH [20,21]. ERK activity was not affected by NH_4Cl, implying that extracellular alkalinization-induced ERK activation is not mediated by intracellular alkalinization. On the other hand, propionate increased phosphorylation of ERK in VSMC (Fig. 2C). These results agree with findings of previous studies that intracellular acidification was a signal that activated ERK [11,22,23].

ERK phosphorylation by extracellular alkalosis was abolished by elimination of extracellular Ca^{2+} due to addition of EGTA but was not affected by verapamil, an inhibitor of VDCC (Fig. 3A). We next carried out an experiment using the $^{45}Ca^{2+}$ uptake method to determine whether Ca^{2+} entry is involved in ERK activation due to extracellular alkalosis. Extracellular alkalosis caused verapamil-sensitive $^{45}Ca^{2+}$ entry, the degree of which was about 50% of that induced by 60 mM KCl (Fig. 3B). The stimulation with KCl, which induces plasmalemmal depolarization [24], also induced verapamil-

sensitive Ca^{2+} entry (Fig. 3B) but did not induce ERK phosphorylation (Fig. 2C). These results suggest that extracellular alkalosis-induced ERK activation is dependent on the presence of extracellular Ca^{2+} but not on transplasmalemmal Ca^{2+} entry.

NADPH oxidase, which produces superoxide anions, has recently been reported to be affected by plasmalemmal H^+ channels in polynuclear leukocytes [16], although the relationship between pH changes and activity of NADPH oxidase is unknown. Moreover, superoxide anions have been shown to activate ERK in VSMC [25]. We thus examined the involvement of NADPH oxidase and oxygen stress in extracellular alkalosis-induced activation of ERK in VSMC. Pretreatment with SOD or Tiron, a radical scavenger, inhibited phosphorylation of ERK stimulated with extracellular alkalosis, suggesting the involvement of a redox-sensitive mechanism in the effect of extracellular alkalosis (Fig. 4A–C). Extracellular alkalosis-induced ERK phosphorylation was also inhibited by DPI, a potent inhibitor of NADPH oxidase (Fig. 4D). Phorbol 12-myristate 13-acetate (PMA), an activator of NADPH oxidase [26], strongly induced phosphorylation of ERK (Fig. 4D). Therefore, extracellular alkalosis, that is, a decrease in extracellular proton concentration, may induce ERK activation via reactive oxygen species that are produced by activation of NADPH oxidase. Further investigation is needed to clarify the relationship between pH changes and NADPH oxidase activity.

The present study is, to the best of our knowledge, the first to demonstrate ERK activation by extracellular alkalosis in VSMC, independently of transplasmalemmal Ca^{2+} entry. Since ERK is a crucial signal for cell proliferation [1], extracellular pH changes may affect proliferation of VSMC via the ERK-related signal cascade.

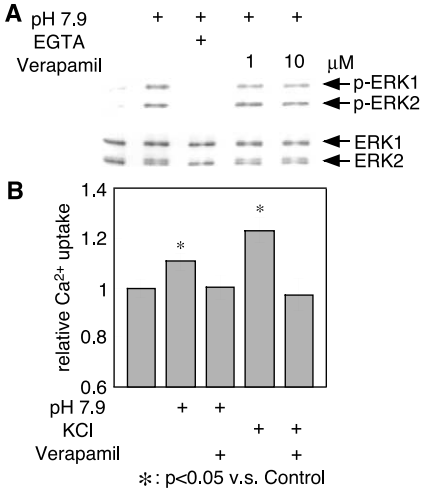

Fig. 3. A: Effects of elimination of extracellular Ca^{2+} and inhibition of Ca^{2+} entry on extracellular alkalosis-induced ERK activation in rat aortic smooth muscle cells. Elimination of extracellular Ca^{2+} and inhibition of Ca^{2+} entry were achieved by the addition of EGTA (3 mM) and verapamil (1 or 10 μM), respectively, to the medium at 30 min before induction of extracellular alkalosis by Tris. B: Effects of Tris and KCl on $^{45}Ca^{2+}$ uptake into rat aortic smooth muscle cells. Verapamil (10 μM) or a vehicle was added to the medium at 30 min before addition of Tris or KCl (60 mM). The results are expressed as the ratio of $^{45}Ca^{2+}$ uptake under different conditions to each control level in the normal medium with pH 7.4. Means ± S.D. of five independent experiments are shown.

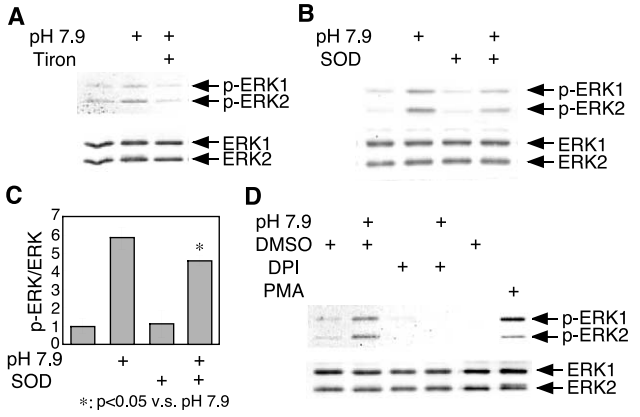

Fig. 4. A: Effects of Tiron, a radical scavenger, on extracellular alkalosis-induced phosphorylation of ERK. Cells were pretreated with Tiron (10 mM) for 30 min before induction of extracellular alkalosis by Tris. Similar results were obtained from at least three separate experiments. B: Effects of SOD on extracellular alkalosis-induced phosphorylation of ERK. Cells were pretreated with SOD (5 U/ml) for 30 min before induction of extracellular alkalosis by Tris. C: Quantitative immunoblot analysis of ERK phosphorylation. Phosphorylated ERK was normalized to the total form and then expressed as relative fold of the phosphorylation level in the condition with extracellular pH 7.4. Means ± S.D. of three independent experiments are shown. D: Effects of DPI, an inhibitor of NADPH oxidase, and PMA, an activator of NADPH oxidase, on ERK phosphorylation. Cells were pretreated with DPI (10 μM) or a vehicle (DMSO, dimethylsulfoxide) for 30 min before induction of extracellular alkalosis by Tris. PMA (100 nM) or vehicle (DMSO) was added to the medium at pH 7.4. Similar results were obtained from at least three separate experiments.

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