MAPKinase and regulation of the sodium–proton exchanger in human red blood cell

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Abstract

The sodium–proton exchanger is activated by various agonists, including insulin, even in human red blood cell. MAPKinase, a family of ubiquitous serine/threonine kinases, plays an important role in the signal transduction pathways which lead to sodium–proton exchanger activation. The aim of our study was to establish the existence of MAPKinase in human red blood cell and to investigate the effects of its activation by insulin and okadaic acid on the sodium–proton exchanger. Immunoblot with antiMAPK antibody revealed the presence of two isoforms, p44ERK1 and p42ERK2. Insulin stimulated MAPKinase activity and increased the phosphorylation of MAPK tyrosine residues, with a peak time between 3 and 5 min. Okadaic acid, an inhibitor of serine/threonine phosphatases, stimulated MAPKinase activity. In the presence of PD98059, an inhibitor of MEK, the upstream activator of MAPKinase, insulin and okadaic acid failed to stimulate MAPKinase. Insulin and okadaic acid increased the activity of the sodium–proton exchanger and this effect was abolished by PD98059. In conclusion, we first describe the presence and activity of MAPKinase in human red blood cell. Furthermore, we demonstrate that in human red blood cell, insulin modulates the sodium–proton exchanger through MAPKinase activation. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Sodium–proton exchanger; Mitogen-activated protein kinase; Insulin; Erythrocyte

1. Introduction

The sodium–proton exchanger is a plasma membrane glycoprotein which mediates the exchange of intracellular H⁺ for extracellular Na⁺ with a 1:1 stoichiometry [1]. Several isoforms of the protein have been identified and are designed as NHE-1 to -5 [2]. The Na⁺/H⁺ exchanger isoform 1 (NHE-1) is an ubiquitous membrane transporter that has an important role in the regulation of cell pH (pHᵢ), cell volume and cell proliferation. NHE-1 is activated by a wide variety of stimuli, including growth factors, phorbol esters, insulin, hormones, and it is regulated by several intracellular kinases such as Ca²⁺-calmodulin, cAMP-dependent kinase, protein kinase C and the mitogen-activated protein kinases (MAPK) [3,4].

The presence of a tightly coupled sodium/hydrogen exchange (NHE) has been well documented in human red blood cells (RBC) [5]. The internal cytosolic amino acid sequence of NHE isoform 1 (NHE1) was demonstrated in human RBC with immunoblot assay [6]. Other NHE isoforms are not expressed in human reticulocytes and RBC [7].
Ceolotto et al. have demonstrated that in human RBC, NHE is activated by phorbol esters and by insulin through different pathways [8]. The phorbol ester PMA stimulates NHE by activation and translocation of PKC from the cytosol to the membrane, while insulin activates NHE by PKC-independent mechanisms. Furthermore, immunoprecipitation assays of NHE-1 in RBC treated with insulin showed an increased NHE-1 phosphorylation [9]. However, the mechanism underlying NHE-1 activation by insulin in RBC is as yet unknown.

Recent studies in vascular smooth muscle cells (VSMC) [10], in platelets [11], and in lymphoblasts [12], have shown that MAPK is involved in NHE-1 regulation. MAPK, a family of ubiquitous Ser/Thr kinases, plays an important role in various signal transduction pathways, including insulin, growth factors, tumor promoters, cytokines, osmotic shock and stress [13]. In mammalian cells, the first and best characterized MAPK isoforms are p44ERK1 and p42ERK2 both of which are essential for intracellular transduction signals of growth factors and insulin [14].

Therefore, the purpose of the present study was to establish the presence of the p44ERK1 and p42ERK2 MAPK isoforms in human RBC, and to describe their role in the regulation of NHE.

2. Materials and methods

2.1. Subject selection

Blood samples were drawn from 11 healthy, lean, normotensive subjects, aged 25-45 years, after an overnight fast.

2.2. Materials

Protease inhibitors, ATP, EDTA, ouabain, neptazane, methazolamide, DIDS, bumetanide, Tris, MOPS, HEPES, MES, nystatin, dithitheitrol, sodium dodecyl sulfate, albumin were purchased from Sigma (St. Louis, MO). MAPK antibodies were purchased from Santa Cruz Biotechnology. Porcine insulin, reagents for the MAPK assay, MAPK-selective substrate were obtained from Calbiochem (La Jolla, CA). [γ-32P]ATP, nitrocellulose membranes were obtained from Amersham (Buckinghamshire, UK). Acutionexx detergent was obtained from American Scientific Products (Boston, MA). The specific MEK inhibitor PD98059 and okadaic acid were from Calbiochem (La Jolla, CA).

2.3. Preparation of red blood cells

Blood was drawn into heparinized tubes and centrifuged at 3000 rpm for 4 min at 4°C. Plasma and buffy coat were removed by aspiration, and red blood cells washed three times with ice-cold washing solution (CWS) that contained (mmol l⁻¹): choline chloride 149, MgCl₂ 1, Tris-MOPS 10, pH 7.4 (4°C).

2.4. Immunoblot analysis of MAPKinase isoforms

Red blood cells were immunoblotted with rabbit polyclonal antibody against MAPK carboxyl-terminal epitope (residues 349-361). RBC were lysed and dissolved in Laemmli buffer [15]. The proteins from RBC were separated by electrophoresis through a 10% polyacrylamide gel. Proteins separated on the gel were electroblotted onto nitrocellulose membrane in blotting buffer containing (mmol l⁻¹): Tris 48, glycine 39, pH 8.3, 0.037% SDS in 20% (v/v) methanol for 2 h at 100 V in the cold, using a Bio-Rad Transblot cell. The nitrocellulose membrane was blocked overnight at 4°C in phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween and 5% bovine serum albumin (BSA). The nitrocellulose membranes were exposed to primary antibody (1:1500 dilution) for p44ERK1 and p42ERK2 overnight at 4°C. The nitrocellulose membranes were washed (four times for 20 min) with the same buffer and then incubated for 1 h at room temperature with 1:4000 goat anti-rabbit antibody conjugated to horseradish peroxidase. Membranes were washed four times with the same buffer, and detection was made using enhanced chemiluminescence system (ECL, Amersham).

2.5. Immunoblot analysis of tyrosine-phosphorylated MAPK

MAPK is active when phosphorylated on both threonine and tyrosine residues. To investigate the effect of insulin on MAPK activation, we used phos-
pho-specific MAPK antibody. Washed RBC were incubated at 37°C in a incubation medium containing (mmol l⁻¹): 150 KCl, 1.0 MgCl₂, 5.0 glucose, 1.0 CaCl₂, 20 Tris-MOPS pH 7.4, albumin 0.1% in the presence or absence of insulin (100 nM). Lysed RBC were electroblotted onto nitrocellulose membranes, and then blocked overnight at 4°C in phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween and 5% bovine serum albumin (BSA), and then immunoblotted with the phospho-specific MAPK antibody (1:1000 dilution). This monoclonal antibody detects only Thr-202-Tyr-204-phosphorylated MAPK.

Detection was made with ECL as described earlier. Blots were scanned and quantified with a Bio-Rad Chemiluminescence Molecular Imaging Systems, and the results were compared to the control readings, on the same blot, set to 100%.

2.6. MAPK activity

The activity of MAPK was assayed by measuring the rate of [³²P]phosphate incorporation from [γ-³²P]ATP using saturating concentrations of the MAPK-selective substrate APRTPGGRRR, derived from bovine myelin basic protein [16]. Washed RBC were incubated for 30 min at 37°C in an incubation medium containing (mmol l⁻¹): 150 KCl, 1.0 MgCl₂, 5.0 glucose 1.0 CaCl₂, 20 Tris-MOPS pH 7.4, albumin 0.1% in the presence or absence of 100 μM PD98059. Thereafter insulin (100 nM for 1, 3, 5, 10 or 15 min) or okadaic acid (1 μM for 20 min) were added to the incubation medium. Cells were lysed with 1 ml of lysis buffer, containing (mmol l⁻¹): Tris-HCl 20, EGTA 2, EDTA 2, sodium fluoride 0.1, sodium orthovanadate 1, PMSF 1, aprotinin 20 μg ml⁻¹, leupeptin 20 μg ml⁻¹, pH 7.5) and were centrifuged at 12 000 rpm for 10 min at 4°C. The standard assay mixture (final volume 40 μl) contained (mmol l⁻¹): Tris-HCl 12.5, pH 7.4, EGTA 2, MgCl₂ 10, PKI (protein kinase A inhibitor) 50 mg ml⁻¹, 0.4 mg ml⁻¹ of peptide, and [γ-³²P]ATP (500 000 cpm/10 μl). Lysates (20–50 μg of protein) were added to this mixture. After 15 min of incubation at 25°C, the reaction was stopped with 10 μl of diluted trichloroacetic acid. A 40-μl aliquot of the reaction media was spotted on a phosphocellulose paper (2.5×2.5 cm) (Whatman P81). The paper was washed four times for 10 min, with stirring, in phosphoric acid, dried, and assayed for ³²P by scintillation counting. MAPK assay was linear for 20 min.

MAPK activity was calculated as difference between the ³²P incorporation in the presence and in the absence of substrate. Data were expressed as pmol of ³²P incorporated into peptide per mg of protein per min.

The protein concentration was measured by the method of Lowry et al. [17], using bovine serum albumin as standard.

2.7. Measurement of NHE

NHE was estimated as net Na⁺ influx driven by an outward H⁺ gradient [8].

2.7.1. Preparation of RBC

Blood was drawn into heparinized tubes and centrifuged at 3000 rpm for 4 min at 4°C. Plasma and buffy coat were removed by aspiration, and the RBC washed three times with ice-cold washing solution (CWS) that contained (mmol l⁻¹): choline chloride 149, MgCl₂ 1, Tris-MOPS 10, pH 7.4 (4°C). The RBC were then resuspended to approximately 50% hematocrit with CWS. Aliquots of this suspension were then used for determination of hematocrit, hemoglobin (optical density at 540 nm), and Na⁺ concentration (atomic absorption spectrophotometry) after appropriate dilution with 0.02% Acationox detergent in double-distilled water.

2.7.2. Modification of cell cation content

The nystatin procedure was used to prepare sodium-depleted cells. All solutions were prepared with deionized, double-distilled water, and the osmolality was checked using a freezing point osmometer. Washed, packed cells were incubated at 15% hematocrit in cold nystatin loading solution (NLS) containing 40 μg ml⁻¹ nystatin for 20 min at 4°C and protected from light. Nystatin was dissolved in dimethyl sulfoxide (5 mg in 1.3 ml). For Na⁺ depletion, NLS contained (mmol l⁻¹): KCl 150, sucrose 50. The cell suspension was then warmed to 37°C for 5 min, and the red blood cells washed four times with warm nystatin washing solution (NWS). NWS had the same composition as the NLS with the pH ad-
justed to 7.4 with 1 mmol l\(^{-1}\) KH\(_2\)PO\(_4\) buffer and 10 mmol l\(^{-1}\) glucose and 0.1% albumin added. Removal of nystatin from the membrane was checked in every experiment by measurements of the passive permeability to Na\(^+\).

2.7.3. Modification of RBC pH

Na\(^+\)-depleted cells (5% hematocrit) were incubated at 37°C for 10 min in acid-loading solutions, which contained (mmol l\(^{-1}\)): KCl 170, MgCl\(_2\) 0.15, ouabain 0.1, bumetanide 0.1 (prepared in dimethyl sulf oxide), glucose 10, and Tris-MES 20 adjusted to pH 6.0 at 37°C. The osmolarity of the acid-loading solutions was adjusted to 360 mOsm by the addition of sucrose. After a 10-min preincubation with the acid-loading solution, 100 µmol l\(^{-1}\) 4,4-diisothiocyanato-stilbene-2,2-disulfonic acid (DIDS) (to inhibit carbonic anhydrase) were added to final concentrations of 100 and 200 µmol l\(^{-1}\), to clamp pH\(_i\). The cells were incubated at 37°C for 20 more minutes and then washed three times with ice-cold pH washing solution containing (mmol l\(^{-1}\)): KCl 170, MgCl\(_2\) 0.15, sucrose 40. After the final wash, the cells were resuspended to approximately 50% hematocrit with acid washing solution and stored on ice until used. Aliquots of the cell suspension were used for determination of hemoglobin, hematocrit, and intracellular Na\(^+\) content. pH\(_i\) was determined with a pH electrode in cell lysate made with 4 vols. of 0.02% Acationox detergent. The cellular Na\(^+\) concentration determined after diluting the lysate with distilled and deionized water. The cation content of the acid loaded cells was expressed per liter original volume, as determined by relating the absorbance at 540 nm of the cell lysate to that of a known volume of red cells. The cell volume was estimated by comparing the hemoglobin per liter of the loaded cells with that of the fresh cells.

2.7.4. Measurement of net Na\(^+\) influx by NHE

NHE was estimated as net Na\(^+\) influx into Na\(^+\) depleted, acid loaded cells driven by an outward H\(^+\) gradient. To this end, initial rates of net Na\(^+\) influx were measured by incubating RBC in two different media, one with pH\(_o\) 8.0 and another with pH\(_o\) 6.0. The difference between both media determines the fraction of Na\(^+\) influx driven by an outward H\(^+\) gradient (i.e. NHE).

The experiment started with the addition of 200 µl of a 50% suspension of the acid loaded cells to 2 ml Na\(^+\) influx media preincubated at 37°C in a shaking water bath. The Na\(^+\) influx media contained (mmol l\(^{-1}\)): NaCl 150, KCl 20, MgCl\(_2\) 0.15, ouabain 0.1, neptazane 0.4, glucose 10, sucrose 40, and either Tris-MOPS 10 (pH 8.0 at 37°C) or Tris-MES 10 (pH 6.0 at 37°C), 360 mOsm l\(^{-1}\). RBC were added to pH\(_o\) 6.0 and pH\(_o\) 8.0 Na\(^+\) influx media. At timed intervals (1, 6 and 10 min for pH\(_o\) 8.0 and 1, 10, and 20 min for pH\(_o\) 6.0) duplicate 250 µl aliquots were pipetted into pre-cooled Eppendorf tubes containing 0.4 ml dibutylphthalate oil layered over 0.7 ml influx wash solution. The transport reaction was terminated by immediate centrifugation at 14,000 x g for 10 s at room temperature. The composition of the influx wash solution was (mmol l\(^{-1}\)): Choline chloride 80, KCl 80, MgCl\(_2\) 0.25, Tris-MOPS 10 (pH 7.4, at 4°C), and sucrose 40. The supernatant was carefully removed by aspiration and the external surface of the tubes wiped dry to eliminate Na\(^+\) contamination. The bottom of the tube, containing the cell pellet, was then cut off and placed into 1 ml of 0.02% Acationox to lyse the cells. The cell lysates were vortexed vigorously and centrifuged at 2000 x g for 5 min at 4°C, and the hemoglobin concentration determined after diluting the lysate with 0.02% Acationox. The lysate Na\(^+\) concentration was determined by atomic absorption spectrophotometry and the cell Na\(^+\) content calculated by relating the optical density of the lysate to the hematocrit and optical density of the fresh cell suspension. Net Na\(^+\) influx was expressed as mmol l\(^{-1}\) cell h\(^{-1}\).

2.7.5. Insulin, okadaic acid and PD98059 incubation experiments

To study the effects of insulin, okadaic acid, PD98059, aliquots of washed RBC were incubated for 30 min at 37°C in an incubation medium containing (mmol l\(^{-1}\)): 150 KCl, 1.0 MgCl\(_2\), 5.0 glucose. 1.0 CaCl\(_2\), 20 Tris-MOPS pH 7.4, albumin 0.1%, in the presence or absence of 100 µM PD98059. Insulin (100 nM) or okadaic acid (1 µM) were then added to the incubation medium for 20 min and to each of the acid loading, sodium depleting and influx media. Sodium depletion, acid load and sodium influx were
conducted according to the same experimental protocol reported above.

2.8. Statistical analysis

The statistical analysis was performed with non parametric tests. Linear regression analysis was also used, when indicated. A P-value < 0.05 was accepted as statistically significant. Data are presented as mean ± standard error (S.E.M.).

3. Results

3.1. Identification and activation of MAPK in human RBC

As shown in Fig. 1, immunoblot analysis revealed the presence of two characteristic bands of 44 and 42 kDa, respectively, corresponding to p44ERK1 and p42ERK2 MAPK isoforms.

MAPK is active when both threonine and tyrosine residues are phosphorylated. To investigate the effect of insulin on MAPK activation we used a phospho-specific MAPK antibody. As shown in Fig. 2, the immunoblot analysis detected the presence of only one band of 44 kDa, corresponding to the phosphorylated MAPK isoform. Quantitative analysis, performed by scanning densitometry, showed that exposure of RBC to 100 nM insulin caused a rapid and transient phosphorylation of MAPK. The maximal increase (48 ± 5%, n = 3) occurred at 3 min, returning to the baseline at 10 min.

We measured MAPK activity in lysates from cells treated with 100 nM insulin, using a specific peptide as a substrate. As shown in Fig. 3, the time course for activation of MAPK by insulin indicated that MAPK activity peaked within 3–5 min, and reached baseline at 10 min.

In order to verify the specificity of insulin-induced MAPK activation, the cells were treated for 30 min with PD98059 (100 μM), the inhibitor of MEK (the kinase capable of MAPK phosphorylation and activation), before being stimulated by insulin. PD98059 completely abolished the effect of insulin on MAPK activity (Fig. 4).

Similar responses were found in RBC treated for 20 min with 1 μM okadaic acid, a serine/threonine phosphatase inhibitor. As shown in Fig. 5, okadaic

Fig. 1. Immunoblot of MAPK in human red blood cells. The first lane (M) is the MW marker, the second (RBC1) and third (RBC2) lanes are samples from two different subjects. MAPK antibody revealed two bands of 44 and 42 kDa, corresponding to p44ERK1 and p42ERK2.

Fig. 2. Immunoblot analysis of phosphorylated-MAPK in the presence of insulin (100 nM) in human red blood cells. The upper panel shows a representative immunoblot with phospho-specific MAPK antibody at control and after 3, 5, 10 min of insulin exposure. The lower graph illustrates the quantitative analysis, performed by scanning densitometry (mean ± S.E., n = 3) expressed as percent of control (taken as 100%). **P < 0.01 versus control.
acid markedly increased MAPK activity (from 0.62 ± 0.13 to 3.02 ± 1.51 pmol min⁻¹ mg protein⁻¹, P < 0.01). PD98059 (100 μM for 30 min) inhibited okadaic stimulation (from 3.02 ± 1.51 to 1.56 ± 0.52 pmol min⁻¹ mg protein⁻¹ P < 0.01) (Fig. 5). On the contrary, Na⁺-depletion with nystatin and cellular acidification did not alter MAPK activity (data not shown).

3.2. Effect of MAPK activation on NHE activity

As previously reported, NHE activity was increased by insulin and okadaic acid [8]. As shown in Fig. 6, we confirmed that insulin (100 nM) and okadaic acid (1 μM) enhance NHE activity from 17 ± 2 to 38 ± 9 mmol l⁻¹ cell h⁻¹, P < 0.05 and 17 ± 2 to 40 ± 7 mmol l⁻¹ cell h⁻¹, P < 0.05, respectively. After incubation with PD98059 (100 μM for 30 min) the effect of insulin and okadaic acid on NHE activity was completely inhibited (Fig. 6).

4. Discussion

Our study demonstrates the presence of MAPK isoforms p44ERK1 and p42ERK2 in human red blood cell. These kinases were activated by insulin and okadaic acid. The insulin-induced MAPK activation seems to be transient, returning to basal levels at 10 min. This effect was completely abolished by PD98059, a specific inhibitor of MEK, the MAPK activator. The activation of MAPK by insulin regulates NHE activity, suggesting a possible link between insulin signaling, MAPK stimulation and NHE-1 activity in insulin-independent red blood cells. This pathway has been already investigated in insulin-dependent cells.

MAPK, a family of ubiquitous Ser/Thr kinases, plays an important role in various signal transduction pathways [13]. The activation of MAPK family is regulated by phosphorylation of tyrosine and threonine residues, both of which are necessary for enzyme activity. Once activated, MAPK are responsible for the phosphorylation and activation of other downstream kinases and regulatory proteins [18]. In particular, two members of this family, the extracellular related kinases 1 (p44ERK1) and 2 (p42ERK2) are involved in the intracellular signaling following insulin stimulation [19]. Recently, it has been shown that MAPK is involved in erythropoietin-induced differentiation. In fact, in human erythroid-forming cells...
erythropoietin and stem cell factor synergistically activate p44ERK1 and p42ERK2 [20].

To our knowledge, this is the first study that documents the presence of p44ERK1 and p42ERK2 in human circulating RBC suggesting that MAPK is present not only in staminal hematopoietic cell, but in all the circulating elements, including RBC and platelets [11]. Although in RBC the physiologic function of MAPK is not clear, our results suggest a potential role of MAPK in the signal transduction mechanisms of these cells.

Insulin receptors have been identified in human RBC [21]. They are about 2000 per erythrocyte, but their function is still unclear. Furthermore, the presence of Ras, a downstream element of the insulin receptor-activated cascade, has already been documented in human RBC [22].

Several studies have shown that p44ERK1 and p42ERK2 are activated by insulin through a Ras-dependent mechanism [19]. Ras is activated by the translocation of Sos to the plasma membrane due to the tyrosine-phosphorylation of Grb2 or IRS-1. Activated Ras recruits RAF which is responsible for phosphorylation and activation of MEK, the upstream kinase of the ERK cascade [23].

In the current study, we demonstrated that in RBC, MAPK is activated by insulin. Phosphorylation of MAPK peaked at 3 min, and its activation was inhibited by PD98059, a specific MEK inhibitor, at the concentration used in our experiments [24]. The activation of MAPK by insulin is transient, probably due to rapid dephosphorylation of the threonine and tyrosine residues by protein phosphatases. In fact, RBC contain a large amount of constitutively active phosphatases [15].

Furthermore, we evaluated the effect of okadaic acid, a serine/threonine protein phosphatase inhibitor, on MAPK activation. Okadaic acid induces a marked increase of serine/threonine residue phosphorylation of various RBC proteins, whereas it does not affect serine/threonine–protein kinase activity [25]. We found that okadaic acid stimulates MAPK activity whose effect is completely abolished by PD98059, suggesting that the latter does not act directly on MAPK, but on MEK or other upstream activators of MAPK activated by RBC phosphatases inhibition.

It has been shown that MAPK cascade is involved in NHE-1 regulation in eukaryotic cell [26]. Pontremoli et al. reported that insulin and okadaic acid increase NHE activity in human RBC [27]. Our data confirm that insulin stimulates NHE activity in RBC. Furthermore, we demonstrate the insulin receptor signaling cascade (i.e. phosphorylation of p44ERK1 and p42ERK2, activation of MAPK activity, activation of NHE) in human RBC. These effects...
were inhibited by PD98059, suggesting a direct linkage between NHE activation and MAPK. MAPK probably does not directly phosphorylate NHE-I [2,10]. Therefore, we hypothesized the existence of other intracellular downstream kinases, capable of phosphorylating the NHE-1.

In conclusion, we found that MAPK isoforms \( p44^{\text{ERK}1} \) and \( p42^{\text{ERK}2} \) play a role in the activation of NHE-1 in human RBC following insulin stimulation. This might account for the correlation between insulin-resistance and cation heteroexchange activity (sodium–proton and sodium–lithium exchange). Further studies are needed to better elucidate such a correlation which is common in patients with essential hypertension [28].

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References


