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Lanthanum induces extracellular signal-regulated kinase phosphorylation through different mechanisms in HeLa cells and NIH 3T3 cells

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Abstract

Lanthanum ion (La^{3+}) was generally regarded as calcium antagonist and was used as calcium channel blocker. However, its potential biological effects on cells were poorly understood. In the present work, it was found that La^{3+} could induce rapid extracellular signal-regulated kinase (ERK) phosphorylation in both HeLa cells and NIH 3T3 cells, but different mechanisms were involved. At a concentration of 30μM or higher, La^{3+} enters the cells and activates ERK through a mechanism involving calmodulin activation inside the cells, which is similar to the action of intracellular Ca^{2+}. However, at lower concentration, free La^{3+} promoted ERK phosphorylation in NIH 3T3 cells outside the cells through an unknown La^{3+} sensing mechanism, while Ca^{2+} exerted much weaker effect. The present results suggested that the biological effects of La^{3+} on cells maybe involve mechanisms beyond calcium antagonist.

Introduction

Lanthanum ion (La^{3+}), a representative of lanthanides, has been regarded as Calcium ion (Ca^{2+}) antagonist (Kuzuya *et al.* 1983; Tang *et al.* 2000; Beedle *et al.* 2002.) due to its similarity in coordination chemistry to Ca^{2+}. Accordingly, La^{3+} was broadly used as a non-specific cellular calcium channel blocker for many years. However, in agricultural application, it was shown that La^{3+} could promote the growth of livestock and production of crops (Ni 2002). In addition, it was also reported that La^{3+} had some potential effects on proliferation (Smith *et al.* 1984; Praeger *et al.* 1989) and apoptosis (Palmer *et al.* 1987; Dai *et al.* 2002) of cells, which suggested that La^{3+} could play other roles beyond calcium channel blocker.

In the response to exotic stimulus, it has been recognized that the mitogen activated protein kinase (MAPK) signal cascade plays a central role, in which extracellular signal-regulated kinase (ERK) pathway is responsible for rapid response to stimulus concerning cell proliferation, development and apoptosis (Chang *et al.* 2003). Once activated, dual phosphorylated ERK (p-ERK) would translocate into nucleus to phosphorylate transcription factors and then regulate gene expression (Johnson *et al.* 2002). In the MAPK signal transduction pathway, certain metal ions had been reported to modulate or participate in the process of signaling (Wu *et al.* 1999; Misra *et al.* 2003). It was shown that La^{3+} could act as a mitogen or an apoptosis inducer, depending on cell type and cultural conditions. Thus, it would be very interesting how La^{3+}, as an exotic stimulus,

could affect on MAPK signal cascade, especially on ERK pathway in various cell lines.

We have previously reported that La^{3+} could induce a rapid phosphorylation of ERK in NIH 3T3 cells, which is related to both La^{3+}-induced cell proliferation and apoptosis (Yu *et al*. 2004). In the present work, the effects of La^{3+} on ERK activation were studied and compared in HeLa cells and NIH 3T3 cells. The experimental results revealed that La^{3+} induced rapid ERK phosphorylation in both cell lines but through different mechanisms, suggesting that La^{3+} could exert more complex biological effects beyond calcium antagonist.

Material and methods

Materials

1,2-Bis(2-aminophenoxy)ethane-N,N,N′, N′-tetraacetic acid tetrakisacetoxymethyl ester) (BAPTA-AM), N-(4-aminobutyl)-5-chloro-2-naphthalene sulfonamide hydro chloride (W13), nitro blue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), Chelex-100, aprotinin and leupeptin were purchased from Sigma. $LaCl_3$ was prepared from La_2O_3 (99.9% in purity). RPMI-1640 Medium, Dulbeccol's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were from GibcoBRL Biotech. Antibodies against phosphorylated ERK (pERK) and corresponding AP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Both HeLa cells and NIH 3T3 cells were obtained from Peking University Health Science Center.

Cell culture and treatment

HeLa cells and NIH 3T3 cells were maintained in RPMI-1640 and DMEM, respectively, supplemented with 10% FBS and 100 units penicillin–100 μg streptomycin per ml at 37 °C in a humidified 5% CO_2 atmosphere. Cells were plated in 6 cm dishes at the density of 1 10^5 cells/ml and cultured for 24 h. Then the medium was replaced with FBS-free medium and cultured for another 12–16 h.

Before incubation with La^{3+} ions, cells were washed three times with Ca^{2+}-free KRH solution (125 mM NaCl, 6 mM KCl, 25 mM Hepes, pH 7.4, 6 mM Glucose, trace amount of Ca^{2+} was removed by passing through a Chelex 100 column). For pretreatments of cell with BAPTA-AM or W13, the agents were added to the desired concentrations and cells were incubated at 37 °C, 5% CO_2 for 1 h (for BAPTA-AM) or 20 min (for W13). Then the cells were incubated with various concentrations of La^{3+} in Ca^{2+}-free KRH solution at 37 °C, 5% CO_2 for either 15 min (for HeLa cells) or 10 min (for NIH 3T3 cells). Then culture medium was aspirated out and 80 μL of cold lysis buffer (100 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.4% SDS, 25 mmol/L Tris–HCl, pH 7.4, 1 mM Na_3VO_4, 10 mM DTT, 1 mM PMSF, 5 μg/mL aprotinin and 5 μg/mL leupeptin) was added and incubated at 4 °C for 10 min. The lysate was collected and the protein concentration was measured using the Lowry method.

Western blot

Aliquots containing 80 μg of total protein were subjected to SDS–PAGE and then electrophoretically transferred to nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in TTBS (125 mmol/L NaCl, 25 mmol/L Tris–HCl, pH 8.0, 0.1% Tween-20) for 2 h, then incubated with primary antibodies in TTBS containing 0.5% non-fat dry milk at 4 °C overnight followed by an incubation with the secondary antibody in the same solution for 1 h. Chromogenic detection of bound antibody was performed with NBT/BCIP staining of alkaline phosphatase. The optical densities of bands were quantified using a Scion Image software.

Results and discussion

Intracellular lanthanum induced ERK phosphorylation in HeLa cells through a mechanism involving calmodulin activation

The effects of La^{3+} on phosphorylation of ERK in HeLa cells were investigated using a monoclonal p-ERK1/2 antibody as described above and the results are shown in Figure 1. It was seen in Figure 1a that La^{3+}, at the concentrations of 30 and 100 μM, promoted ERK phosphorylation in KRH solution, but did not at lower concentration (3 and 10 μM). To clarify whether extracellular or intracellular La^{3+} pro-

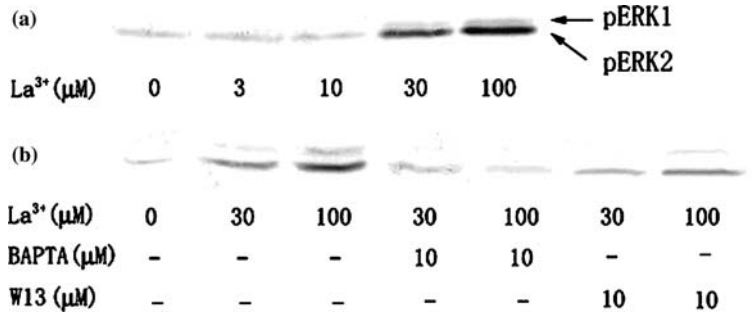

Figure 1. Phosphorylation of ERK in HeLa cells induced by La^{3+}. (a) Effect of different concentrations of La^{3+} on ERK phosphorylation, (b) The effects of BAPTA-AM and W13 on the promotion effects of La^{3+} on ERK phosphorylation. Indicated concentrations of La^{3+} were added to cells pretreated by BAPAT-AM or W13 as described in Material and methods. Aliquots (80 μg total protein/lane) of whole cell lysates were applied to 10% SDS–PAGE. After transferring protein to a nitrocellulose membrane, the phosphorylated form of ERK1 (44 kD) /ERK2 (42 kD), indicated by arrows, were stained immunologically.

duced such effect, BAPTA-AM, an intracellular metal ion chelator, was loaded to the cells to eliminate the effects of intracellular La^{3+}. As shown in Figure 1b, pretreatment of HeLa cells with BAPTA-AM abolished the promotion effect of La^{3+} on ERK phosphorylation. It was also observed that W13, a specific calmodulin inhibitor, could also inhibit this effect partially. These results suggested that La^{3+}, after getting into HeLa cells somehow, promoted the ERK phosphorylation intracellularly. Moreover, activation of calmodulin could be involved in the mechanism of ERK activation.

To confirm the above postulation, a calcium ionophore, A23187, was employed to introduce La^{3+} into HeLa cells. A23187 has previously been proved to efficiently accelerate La^{3+} transportation into cells (Amellal *et al.* 1983). The results were shown in Figure 2 and the effect of Ca^{2+} was monitored for comparison, since it was known that increased intracellular Ca^{2+} activates calmodulin and thus results in ERK phosphorylation (Enslen *et al.* 1996; Abraham *et al.* 1997; Agell *et al.* 2002). It can be seen in Figure 2a, as well as in Figure 2b, that in the presence of A23187, both Ca^{2+} (5 mM) and La^{3+} (10 μM) promoted ERK phosphorylation remarkably. In contrast, extracellular La^{3+} and Ca^{2+} at the same concentrations failed to work in the absence of the ionophore. It is noted that, in the absence of Ca^{2+} and La^{3+}, A23187 could promote ERK phosphorylation to a little extent on its own

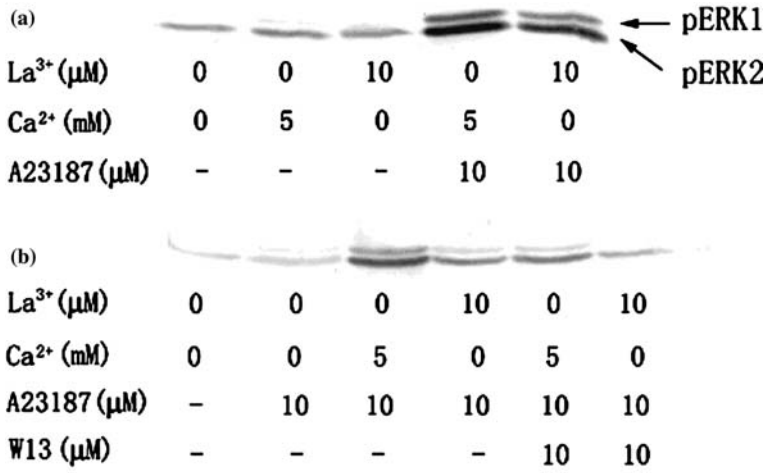

Figure 2. The effects Ca^{2+} and La^{3+} on ERK phosphorylation in HeLa cells. (a) The comparison between the effects of extracellular metal ions on ERK phophorylation and those of intracellular metal ions introduced by calcium ionophore, A23187, (b) Effect of W13 on ERK phosphorylation induced by intracellular Ca^{2+} and La^{3+} introduced by A23187. Details see the Materials and methods.

(Figure 2b), which could be the result of the increase of intracellular Ca^{2+} presumably because of A23187-induced release of Ca^{2+} from intracellular calcium store. Again, the calmodulin inhibitor, W13, was observed to inhibit phosphorylation of ERK (Figure 2b) as well. The present results showed that intracellular La^{3+}, after getting into cells by ionophore or some other ways, could promote ERK phosphorylation in a way partially dependent on the calmodulin activation, in which La^{3+} seemed to act like Ca^{2+}.

These results are consistent with the previous studies *in vitro*, in which it has been shown that La^{3+} could bind to calmodulin with high affinity either in presence or absence of calmodulin binding peptide/proteins (CaMBPs) (Buccigross *et al.* 1986; Hu *et al.* 2004) and activated it like Ca^{2+} (Sotiroudis *et al.* 1986). Further, the present results provided solid evidences that calmodulin would be one major target *in vivo* for La^{3+}.

It is interesting that how La^{3+} in concentration of 30 μM or higher could permeate into the Hela cells and then activate calmodulin. Although La^{3+} has previously been regarded as a membrane non-permeable metal ion, there were large numbers of evidences suggesting that Lanthanide ions, could permeate into cell through multiple pathways (Powis *et al.* 1994; Du *et al.* 2002). It is possible that La^{3+} could get into HeLa cell via the self-assistant diffusion mechanism, in which binding of La^{3+} ions to cell membrane induces structure changes of the phospholipid bilayer, thus increasing the permeability of membrane (Cheng *et al.* 1999a, b).

Extracellular La^{3+} promoted ERK phosphorylation of NIH 3T3 cells through an unknown metal ion sensing mechanism

As indicated above, La^{3+} at the concentration of 10 μM or less could not promote ERK phosphorylation in HeLa cells. On the contrary, it did result in ERK phosphorylation in NIH 3T3 cells (Figure 3a). Moreover, activation of ERK was found not to be inhibited by either intracellular chelator BAPTA or calmodulin inhibitor W13, suggesting that activation of ERK in NIH 3T3 should be in quite different manner from that in HeLa cells.

To find out whether La^{3+} ions act outside of the cells, apo-calmodulin was added in Ca^{2+}-free KRH solution to arrest the extracellular La^{3+}. Camodulin binds La^{3+} with high affinity ($K_d \sim 10$ nM) (Buccigross *et al.* 1986) and is not permeable to cytoplasmic membrane. The results in Figure 3b showed that activation of ERK by La^{3+} decreased with the increase of apo-calmodulin concentrations. These results clearly suggested that the extracellular free La^{3+} ions were responsible for the promotion of ERK phosphorylation.

It has been reported that extracellular Ca^{2+} could promote ERK phosphorylation through a calcium-sensing receptor (CaR), and lanthanide ions were also found to bind to CaR and result in

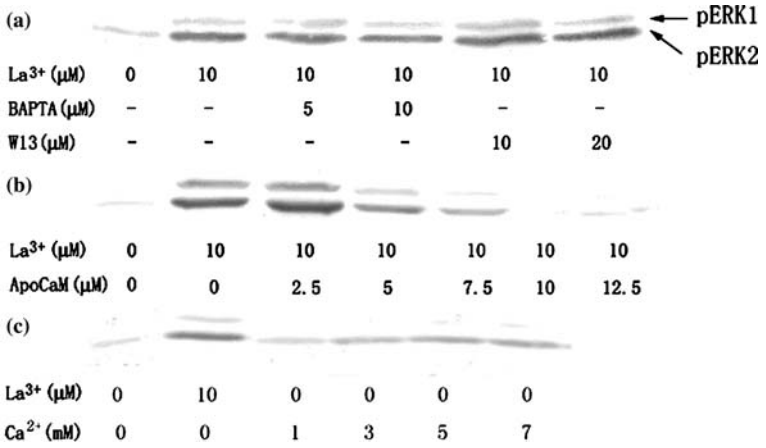

Figure 3. The ERK phosphorylation in NIH 3T3 cells induced by La^{3+}. (a) ERK phosphorylation upon incubation with La^{3+} and the effects of BAPTA and W13 on it. (b) The effects of apo-calmodulin, a non-permeable La^{3+} chelator, on the promotion effect of La^{3+} on ERK phosphorylation, (c) The effects of Ca^{2+} on ERK phosphorylation in NIH 3T3 cells. The process was the same as that in the case of La^{3+}, except the replacement of La^{3+} by Ca^{2+}.

ERK activation (Shorte *et al*. 1996; Hobson *et al*. 2003). However, NIH 3T3 cells were previously shown not to express CaR (Canaff *et al*. 2001). Although Ca^{2+} at concentrations over 3 mM could result in ERK phosphorylation in NIH 3T3 cells, but the extent was much smaller than that in the case of La^{3+} (Figure 3c). Therefore, there might be in NIH 3T3 cells a novel extracellular metal sensing mechanism accounting for La^{3+}-promoted phosphorylation of ERK, which is being under further investigation in our laboratories.

In summary, our experimental data showed that La^{3+} could promote phosphorylation of ERK by two completely different pathways in different cell lines. In HeLa cells, La^{3+} at concentrations over 30 μM could permeate membrane and function as Ca^{2+} to activate calmodulin, then cause ERK phosphorylation, however, in NIH 3T3 cells, the extracellular free La^{3+} ions could result in activation of ERK from outside through a putative La^{3+} sensing mechanism. The later effects in NIH 3T3 cells might suggest novel biological functions of La^{3+} less or not related to calcium.

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