La^{3+}-Induced Extracellular Signal-Regulated Kinase (ERK) Signaling via a Metal-Sensing Mechanism Linking Proliferation and Apoptosis in NIH 3T3 Cells†

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ABSTRACT: The effects of La^{3+} on the extracellular signal-regulated kinase (ERK) signaling were investigated to explore the mechanism by which La^{3+} results in cell proliferation associated with apoptosis in mouse embryo fibroblast NIH 3T3 cells. Our data showed that La^{3+} ions could induce a pulse of phosphorylation of ERK mainly through an unknown metal-sensing mechanism, which is different from the Ca^{2+}-sensing receptor . The putative sensor protein showed one binding site for La^{3+} with a dissociation constant of ∼8 nM. Inductions of c-fos, c-myc, and cyclin D1 and phosphorylation of retinoblastoma protein (pRb) were observed after activation of ERK. These results are consistent with our previous observation that La^{3+} promotes proliferation by helping the cells pass through the G1/S restriction point and enter S phase. This La^{3+}-induced signaling cascade exhibited abnormally sustained *c-myc* induction and pRb phosphorylation. Furthermore, a continual increase of the p53 level was observed along with the signal transduction, and a significant decrease of B-cell lymphoma/leukemia-2 gene was observed after ∼18 h of incubation. All of the results were highly correlated with the increase of S-phase population and apoptotic cells. Therefore, the experimental results suggested that La^{3+} induced cell proliferation and apoptosis compatible to a p53-related mechanism in NIH 3T3 cells via an ERK-signaling cascade induced by a metal-sensing mechanism.

Lanthanide (Ln)[1] is a group of metal ions with similar properties, which have been known for their diversity in biological effects (*1*, *2*) and great potential in medical applications (*3*). Gd^{III} compounds have been widely used as a diagnostic contrast medium (*4*). Recently, lanthanum has been proposed as a phosphate binder for the treatment of hyperphosphatemia of chronic renal failure (CRF), but CRF enhanced tissue lanthanum accumulation (*5*). In Chinese agricultural practices, Ln compounds have been used to increase the production of crops and promote the growth of livestock for many years (*6*). The physiological effects of Ln related to their profound effects on cell proliferation and apoptosis are also of particular interest and medical significance (*3*, *7*, *8*).

There have been many works on the effects of Ln on cell proliferation or apoptosis (*7*−*11*), varied by cell lines, Ln species, and the concentrations of Ln. Among Ln members, La^{3+} is of considerable interest and has been used extensively as a calcium-channel blocker or antagonist in the studies of cellular or other biological systems (*12*, *13*).

The mechanisms of Ln affecting cell proliferation/apoptosis have been suggested to relate to their effects on cellular signal transduction. One major expected target of Ln is the Ca^{2+}−calmodulin (CaM) system (*14*, *15*). In many studies, Ln^{3+} was shown to inhibit Ca^{2+} influx (*13*). However, Ln^{3+} was found to activate a Ca^{2+}-sensing receptor (CaR) and induce an increase of the cellular Ca^{2+} concentration (*16*). It was also shown that Ln^{3+} could weakly stimulate the hydrolysis of phosphoesters including phosphatidylinositols (PIs) and was expected to result in downstream events along the routes of IP_3/Ca^{2+} and DAG/protein kinase C (PKC) signaling systems (*3*, *17*); Incubation of hepatocytes with Ce^{3+} increased the cyclic adenosine monophosphate (cAMP) content, presumably by activating adenylate cyclase, but chemical studies showed that Ce^{3+} promotes the hydrolysis of cAMP (*3*). We previously reported that La^{3+} resulted in cell proliferation associated with apoptosis in NIH 3T3 mice

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[1] Abbreviations: Ln, lanthanide; CaM, calmodulin; CaR, Ca^{2+}-sensing receptor; PI, phosphatidylinositol; ERK, extracellular signal-regulated kinase; CDK, cyclin-dependent kinase; pRb, retinoblastoma protein; FBS, fetal bovine serum; BAPTA/AM; 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester); fluo-3/AM, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)-phenoxy]-2-[2-amino-5-methylphenoxy]ethane-*N*,*N*,*N*^{9},*N*^{9}-tetraacetic acetoxymethyl ester; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; Bcl-2, B-cell lymphoma/leukemia-2 gene; TG, tharpasigargin; PKC, protein kinase C; LSCM, laser-scanning confocal microscopy.

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embryo fibroblasts; both processes shared a key point of phosphorylation of extracellular signal-regulated kinase (ERK) induced by La^{3+}, suggesting the important impact of La^{3+} on ERK signaling (*18*).

It was well-recognized that, in eukaryotic cells, the ERK cascade controls cell proliferation via cell-cycle machinery (*19*). ERK is activated by phosphorylation, which is triggered by either a receptor (*20*) or an increase of intracellular Ca^{2+} through a CaM-dependent pathway (*21*). Upon activation, ERK translocates into the nucleus, where it induces transcription factors including proto-oncogenes such as *c-fos*, *c-myc*, and cell-cycle activators such as D-type cyclins (*22*). Cyclin D1 activates cyclin-dependent kinases (CDKs) by forming cyclin D1/CDK4/6 complexes and in turn causes phosphorylation of retinoblastoma protein (pRb) in collaboration with cyclin E/CDK2 complexes, thus resulting in dissociation of pRb from E2F transcription factors (*23*, *24*). Expression of cyclin D1 is essential for the cell to pass the G1/S restriction point to launch DNA synthesis (*25*).

On the other side, there are elaborate mechanisms that control the balance between cell growth/division and programmed cell death (*24*, *26*). It was proposed that expression of c-myc might couple proliferation with the induction of apoptosis by several mechanisms (*27*−*33*); inappropriate activation of *c-myc* may promote or sensitize cells to apoptosis. Survival of the cells with deregulated c-myc expression needs a continuous supply of survival factors or additional anti-apoptotic factors, for example, elevated expression of the B-cell lymphoma/leukemia-2 gene (Bcl-2) or defects in the p53 pathway (*34*). pRb/E2F was also shown to connect with p53 accumulation and pathways that control apoptosis (*35*). The deficiency of pRb and/or overexpression of E2F1 were demonstrated to induce apoptosis under conditions where serum growth factors, which normally impart survival signals, are limiting (*36*). It has been suggested that the coupling mechanisms could be helpful in preventing uncontrolled proliferation (*24*, *26*, *37*).

In the present work, the effects of La^{3+} on the ERK and related cellular signaling, including changes of the intracellular Ca^{2+} level, were investigated in NIH 3T3 cells. The experimental data showed that La^{3+} caused ERK phosphorylation mainly through an unknown metal-sensing mechanism. The time course of activation of ERK and related signals was monitored and found that La^{3+} resulted in the induction of cyclin D1, extended expression of c-myc, and lasting phosphorylation of pRb. Apoptosis of NIH 3T3 cells was observed along with the increase of p53 and decrease of Bcl-2 proteins. On the basis of the experimental results, the mechanisms by which La^{3+} promoted proliferation associated with cell apoptosis in NIH 3T3 cells are discussed.

MATERIALS AND METHODS

Materials. Dulbeccol's modified Eagle's medium (DMEM) was obtained from GibcoBRL; fetal bovine serum (FBS) was purchased from Hyclone; trypsin, nifedipine, flunarizine, thapsigargin (TG), 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM), W13 (*N*-(4-aminobutyl)-5-chloro-2-naphthalene sulfonamide hydrochloride), and Chelex 100 were purchased from Sigma; and membrane-permeable calcium probe, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-[2-amino-5-methylphenoxy]ethane-*N*,*N*,*N*^{9},*N*^{9}-tetraacetic acetoxymethyl ester (fluo-3/AM), was purchased from Molecular Probes. Rabbit polyclonal antibodies against c-myc and phosphor-pRb (S780) were bought from Cell Signaling Technology. Mouse monoclonal antibodies against phosphorylated ERK (pERK) and cyclin D1, rabbit polyclonal antibodies against ERK, pRb, c-fos, Bcl-2, and p53, goat polyclonal antibodies against actin, horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG, goat anti-rabbit IgG, and the enhanced chemiluminescence (ECL) reagents were obtained from Santa Cruz Biotechnology. Other reagents were of analytical grade. La^{3+} (lanthanum chloride) solution was prepared from lanthanum oxide (purity > 99.9%).

Purification of chicken CaM and preparation of apoCaM were carried out according to the procedures previously described (*14*).

Cell Cultures. NIH 3T3 cells, a mouse embryo fibroblast cell line, were obtained from Peking University Health Science Center and cultured in DMEM supplemented with 10% FBS and 100 units of penicillin−100 μg of streptomycin/mL at 37 °C in a humidified 5% CO_2 atmosphere. All experiments were performed using cells within 30 passages.

Western Blotting. Cells were plated in Petri dishes and cultured until they reached 80−90% confluence, and then the culture medium was replaced with serum-free DMEM. After cells were incubated for ~12 h, they were washed by modified KRH solution [125 mmol/L NaCl, 6 mmol/L KCl, 6 mmol/L glucose, and 25 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7.4, passed through a Chelex 100 column to remove trace high-valence metal ions] and then incubated with media containing desired concentrations of La^{3+} or other reagents/drugs for the desired time as described in the Results. Then, cells were lysed in cold TGH buffer [1% Triton X-100, 0.4% sodium dodecyl sulfate (SDS), 10% glycerol, and 50 mM HEPES at pH 7.2] supplemented with 100 mM NaCl and protease and phosphatase inhibitors (5 μg/mL aprotinin, 5 μg/mL leupeptin, 100 mM PMSF, 1 mM Na_3VO_4, 50 mM NaF, and 1 mM β-phosphaglycerol). The lysate were clarified by centrifugation (13000*g* at 4 °C for 10 min), and the protein concentration of the lysate was determined by using the Lowry method. Aliquots containing 80 μg in total protein were subject to SDS−PAGE and electrophoretically transferred to a nitrocellulose membrane. The membrane were blocked with 10% nonfat dry milk in TBS (Tris-balanced saline; 50 mM Tris-HCl and 150 mM NaCl at pH 7.5) for 2 h, then blotted with appropriate primary antibodies (mouse monoclonal antibodies against pERK, c-myc, and cyclin D1; rabbit polyclonal antibodies against ERK, pRb, phosphor-pRb (S780), c-fos, Bcl-2, and p53; and goat polyclonal antibody against actin) in 5% bovine serum albumin (BSA)/TTBS (0.02% Tween 20 in TBS) at 4 °C overnight, and then incubated with an IgG in 5% BSA/TTBS for another 1 h. Bound antibody was detected with HRP-labeled corresponding secondary antibodies using an enhanced chemiluminescent method according to the protocol provided by the manufacturer. The optical densities of bands were quantified by a Scion Image software.

Effects of Extracellular Ca^{2+} and Intracellular Ca^{2+} Modulators on La^{3+}-Induced ERK Phosphorylation. NIH 3T3 cells plated in Petri dishes were cultured with 100 μM La^{3+} in serum-free DMEM or in Ca^{2+}-free KRH buffer for 24 h, or the cells were pretreated with TG (1 μM/15 min), which

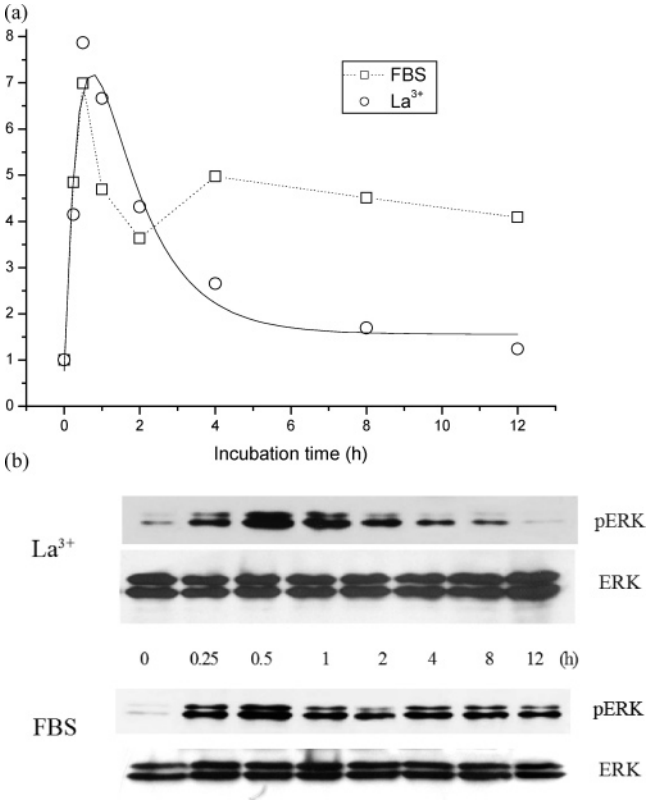


FIGURE 1: Temporal change of ERK1/2 phosphorylation upon incubation of NIH 3T3 cells with La^{3+}. Cells were cultured in serum-free DMEM for 12 h, followed by incubation with 100 μM La^{3+} (○, —) or 10% FBS (□, ···) for the indicated time. After that, the cells were lysed and phosphorylated ERK was detected (b) as described in the Materials and Methods. The blots were quantified, and the data were fitted into a biphasic exponential decay model with $r^2 = 0.952$ (a).

deplete intracellular Ca^{2+} pools, or intracellular Ca^{2+} chelator, BAPTA/AM (10 μM/30 min), and then exposed to La^{3+} for 30 min. Then, the cells were lysed, and phosphorylated ERK was detected by Western blotting as described above.

Measurement of Intracellular Ca^{2+}. Cells plated in Petri dishes were incubated with serum-free DMEM medium containing 5 μM fluo-3-AM for 45 min in the dark and then washed twice with serum-free medium and incubated with KRH buffer containing 1 mM $CaCl_2$. The intracellular Ca^{2+} level was monitored by recording the fluorescence on laser-scanning confocal microscopy (LSCM) with excitation at 488 nm and an emission filter of LBP530, while desired concentrations of La^{3+}were added. Alternatively, cells were collected by trypsic digestion, and fluorescence measurements of the intracellular Ca^{2+} level were performed on a cytometer (FACScan, BD Bioscience, San Jose, CA) with excitation at 488 nm and emission at 543 nm; 20 000 cells were analyzed for each sample.

RESULTS

La^{3+} Induces ERK Phosphorylation. Incubation of NIH 3T3 cells with 100 μM La^{3+} resulted in a rapid wave of phosphorylation of ERK1/2. Figure 1 shows the temporal change of ERK2 phosphorylation upon incubation of NIH 3T3 cells with La^{3+}. Data could fit into the biphasic exponential decay model ($r^2 = 0.952$) with the time constants of 0.6 ± 0.2 h for both rising and dropping phase,

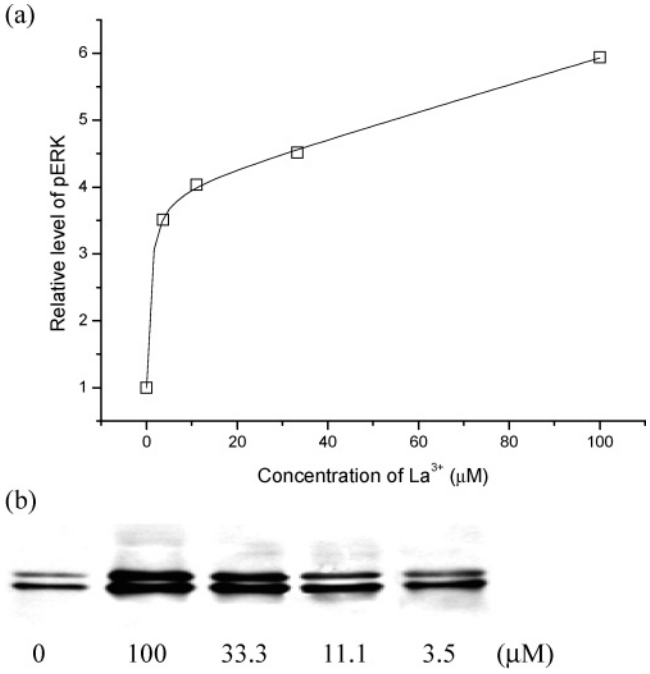


FIGURE 2: Concentration dependency of ERK1/2 phosphorylation induced by La^{3+}. Cells were serum-starved for 12 h, followed by incubation with various concentrations of La^{3+} for 30 min. Then, the phosphorylated ERK1/2 was detected (b) and quantified, and the data were fitted into a nonspecific binding model with $r^2 = 0.999$ (a).

respectively. In contrast, NIH 3T3 cells incubated in 10% FBS medium gave two waves of ERK phosphorylation with comparable intensity as that induced by La^{3+} (Figure 1). The first wave overlapped with that induced by La^{3+}, and the second wave was around 4−6 h.

Figure 2 shows the concentration dependency of ERK2 phosphorylation induced by La^{3+} in culturing medium. Data were found to fit into one site with the nonspecific binding model ($r^2 = 0.999$) with an apparent dissociation constant of 0.77 ± 0.20 μM.

The apparent parameters for La^{3+} ions to activate ERK phosphorylation were estimated by incubation of NIH 3T3 cells with La^{3+} in the presence of various concentrations of malic acid or apoCaM as competitive ligands in the calcium-free KRH solution as described in the Materials and Methods. With the increase of the concentration of malic acid, the degrees of ERK phosphorylation reduced gradually, suggesting that free La^{3+} ions would be the effective species. The concentrations of free La^{3+} were calculated according to the binding constants of La^{3+} to malic acid. As shown in Figure 3, The degree of ERK2 phosphorylation was plotted as the function of free La^{3+} concentration. Data fitting using the Hill model revealed that the activation constant (i.e., apparent dissociation constant K_d on the putative target activating ERK phosphorylation) was 7.5 ± 0.9 nmol/L with a Hill coefficient (n) of 0.86 ± 0.12, indicating only one type of La^{3+}-binding sites. Identical results ($K_d = 8.3 \pm 0.9$ nmol/L) were also obtained using apoCaM as the competitive ligand (data not shown).

La^{3+} Promotes Expression of c-fos. The temporal change of expression of immediate early gene *c-fos* upon incubation with 100 μM La^{3+} was examined by Western blotting as described in the Materials and Methods. As shown in Figure 4, La^{3+} induced a transient but significant increase of c-fos

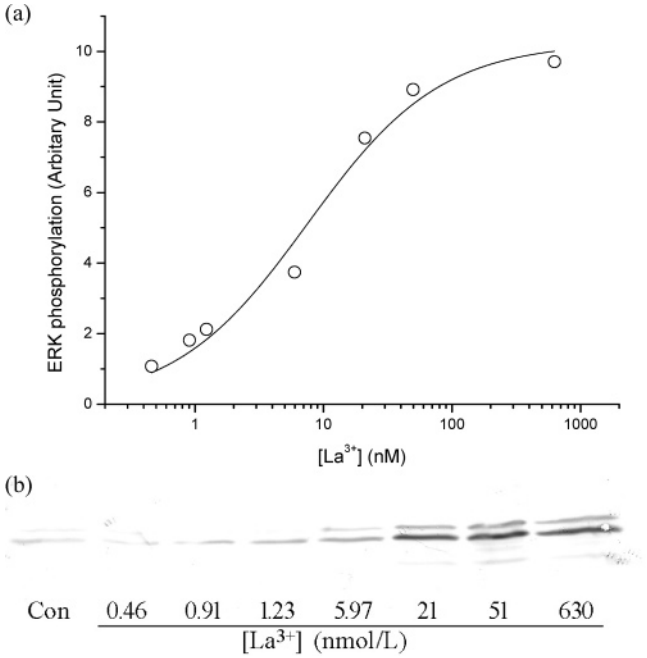


FIGURE 3: Free La^{3+} concentration dependency of ERK1/2 phosphorylation induced by La^{3+}. Cells were starved and exposed to 100 μM La^{3+} in the presence of various concentrations of malic acid in KRH buffer, and then the phosphorylated ERK1/2 was detected as described in the Materials and Methods (b). The free La^{3+} concentrations were calculated according to the binding constants of La^{3+} to malic acid. The blots were quantified and plotted against the free La^{3+} concentration, and then the data were fitted into a Hill model with a dissociation constant (K_d) of 7.5 $\pm$ 0.9 nmol/L and a Hill coefficient (n) of 0.86 $\pm$ 0.12 (a).

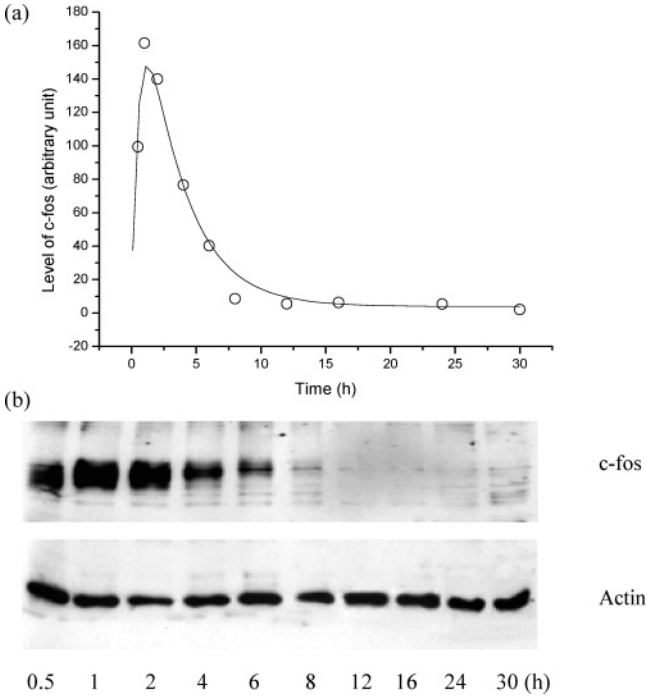


FIGURE 4: La^{3+}-induced expression of c-fos protein in NIH 3T3 cells. The cells were serum-starved for 12 h, followed by incubation with 100 μM La^{3+} for the indicated time, and then cellular c-fos protein was detected as described in the Materials and Methods (b). The blots were quantified and plotted against La^{3+} incubation time, and then the data were fitted into a biphasic exponential decay model with $r^2 = 0.977$ (a).

protein expression. Data fitting to the biphasic exponential decay model ($r^2 = 0.977$) gave the time constants of 0.6 $\pm$ 0.4 and 3.1 $\pm$ 0.7 h for the rising and dropping phase, respectively; this indicates a peak of c-fos expression at the time of about 1.7 h upon incubation with La^{3+}.

La^{3+} Promotes Expression of c-myc, Cyclin D1, and p53. The effects of La^{3+} on the expression of genes *c-myc*, *cyclin D1*, and *p53* were examined by Western blotting as described in the Materials and Methods, and the results were shown in Figure 5. La^{3+} induced two waves of expression of c-myc protein (•••, Figure 5a), which were peaked at ~ 7 and 24 h, respectively. In addition, the immunofluorescence staining (data not shown) revealed that the c-myc protein was mainly located in the nucleus, indicating it action as an active transcription factor. The expression of cyclin D1 gave one peak around 11 h (—, Figure 5a). The expression of p53 seemed to start after pERK/c-fos waves, increase continually, and reach the maximum after ~18 h.

Interestingly, the first c-myc peak, the cyclin D1, and the maximum level of p53, although appearing at different time periods, all exhibited a similar dependency on the concentrations of La^{3+} (Figure 5b, $r^2 = 0.993$), which shares an EC_{50} of 15.8 $\pm$ 0.6 μM and a Hill coefficient $n = 1.0 \pm 0.2$.

The cell-cycle distribution upon incubation with La^{3+} for 24 h was analyzed by flow cytometry; the percentage of S-phase population was calculated to be 9.88, 21.27, 24.06, 28.83, and 36.56% for 0, 3.7, 11.1, 33.3, and 100 μM La^{3+}, respectively. In contrast, the S-phase population stimulated by 10% FBS was 32.21%. The correlations between the expression of c-myc, cyclin D1, and p53 and S-phase population were estimated by plotting the levels of protein expression against the percentage of S-phase population under each concentration of La^{3+}. An overall correlation coefficient (r^2) was calculated to be 0.982.

La^{3+} Promotes Phosphorylation of Rb. Figure 6 shows the time course of phosphorylation of Rb protein upon incubation with 100 μM La^{3+}. The hyperphosphorylated Rb protein band immigrated as a 110 kDa band, while the hypophosphorylated form is around 105 kDa. The La^{3+}-induced pRb phosphorylation was further confirmed by detecting phosphor-pRb (S780), which is phosphorylated by cyclin D1/CDK4 (*38*), using a specific antibody. La^{3+} induced at least one wave of pRb phosphorylation, which peaked at ~8 h. The level of pRb phosphorylation increased again after ~18 h.

La^{3+} Results in a Decrease of the Bcl-2 Level. The levels of Bcl-2, which extensively participate in apoptosis regulation, were determined by Western blotting as described in the Materials and Methods. As shown in Figure 7, data fitting to the Boltzmann sigmoidal model ($r^2 = 0.861$) revealed that the level of Bcl-2 sharply decreased during the hours from ~18 to 28 h upon La^{3+} incubation.

Effects of La^{3+} on the Intracellular Ca^{2+} Level. The effects of La^{3+} on the intracellular Ca^{2+} level were analyzed on a LCM as described in the Materials and Methods. As shown in Figure 8, La^{3+} did not cause immediate elevation of the intracellular Ca^{2+} level at the concentration of 10 μM. However, 100 μM La^{3+} could result in a significant increase of intracellular Ca^{2+}. Meanwhile, as shown by the inset of Figure 8, 10 μM La^{3+} could induce ERK phosphorylation, thus excluding the possible influence of collagen on the promotion effects of ERK phosphorylation. The intracellular Ca^{2+} elevation induced by 100 μM $La^{3+}$was found to be inhibited by pretreatement with nifedipine (5 μM, 10 min)

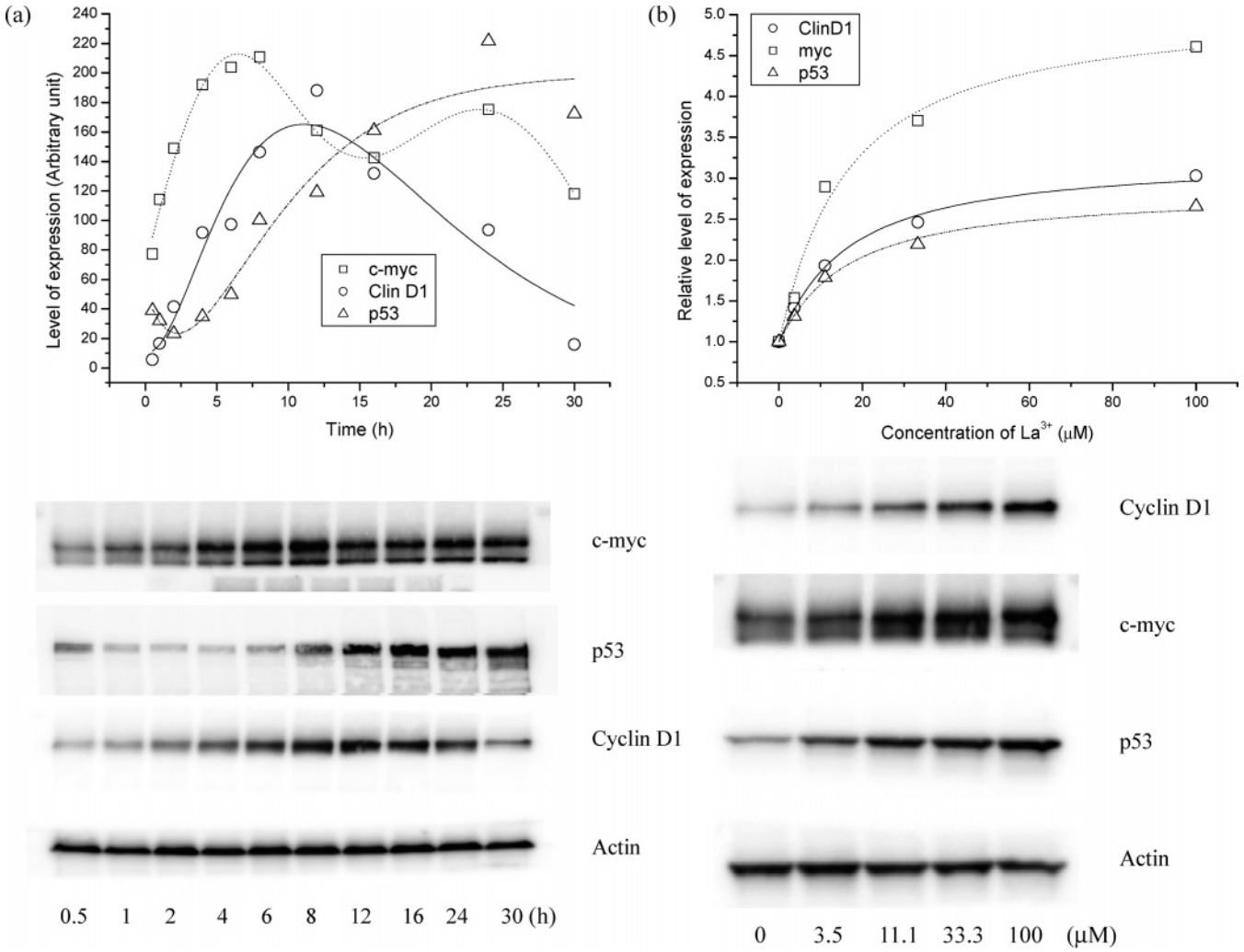


FIGURE 5: Time- and concentration-dependent expression of protein c-myc, cylin D1, and p53 upon incubation of NIH 3T3 cells to La^{3+}. Cells were serum-starved for 12 h, followed by incubation with 100 μM La^{3+} for the indicated time or with indicated concentrations of La^{3+} for 24 h, and then the cellular c-myc, cylin D1, and p53 protein were detected and quantified. The data were plotted against incubation time (a) and La^{3+} concentration (b).

but not affected by flunarizine (5 μM, 10 min) or TG (1 μM, 15 min) (data not shown). Removal of extracellular Ca^{2+} could also abolish the La^{3+}-induced elevation of intracellular Ca^{2+}.

Effects of Ca^{2+} and Modulators on La^{3+}-Induced ERK Phosphorylation. To explore whether cellular Ca^{2+} could be involved in La^{3+}-induced phosphorylation of ERK, the NIH 3T3 cells were preincubated in Ca^{2+}-free KRH buffer or pretreated with BAPTA/AM (an intracellular Ca^{2+} chelator) or TG (depletion of intracellular Ca^{2+} stores) as described in the Materials and Methods. As shown in Figure 9, none of these treatments exhibited significant effects on La^{3+}-induced ERK phosphorylation. However, all of these treatments resulted in a severe loss of cell viability (data not shown).

DISCUSSION

La^{3+} Initiates ERK Phosphorylation through a Novel Metal-Sensing Mechanism. The ERK cascade is one of the most frequently studied signaling systems, which is known to control the expression of various cell-cycle regulators and participate in multiple cellular functions, such as proliferation (*39*), differentiation (*40*), survival, and apoptosis (*19*). Hence, we observed the activation of ERK and its downstream events to explore the mechanism by which La^{3+} influences the cell growth.

Phosphorylation of ERK can be initiated by extracellular growth signals coming from a receptor or intracellular Ca^{2+} in a CaM-dependent manner (*41*). In addition, intracellular Ca^{2+} may also activate ras-mediated ERK signaling in some types of cells or induce the expression of mitogen-activated protein kinase (MAPK) phosphatase, MKP, and lead to the deactivation of ERK (*21*, *42*).

In NIH 3T3 cells, as shown in Figures 1 and 2, La^{3+} induced ERK phosphorylation in a time- and concentration-dependent manner. The concentration dependency (Figure 2) of ERK phosphorylation indicated that ERK is activated in both nonspecific and specific binding ways. The activation by nonspecific binding made a much smaller contribution and only becomes significant at a high concentration of La^{3+}. More important is the activation manner by specific binding to a certain cellular target, which exhibited an apparent half-activation concentration of 0.77 ± 0.20 μM. Although La^{3+} ions at tens of micromolar concentrations were able to enter the cell via self-assistant diffusion and other pathways (*43*), the action of La^{3+} inside cytoplasm should be excluded

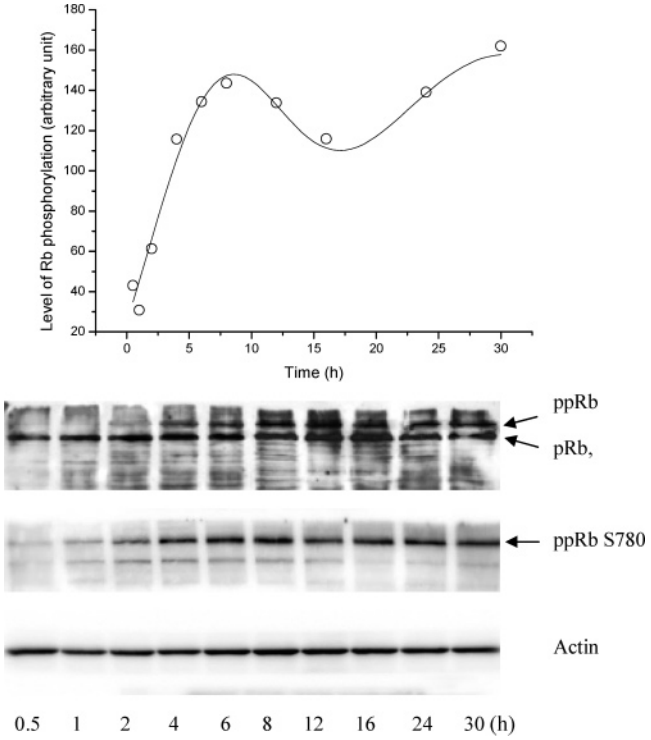


FIGURE 6: Time course of phosphorylated Rb protein upon incubation with 100 μM La^{3+}. Cells were serum-starved for 12 h followed by incubation with 100 μM La^{3+} for the indicated time, and then phosphorylation of the cellular Rb protein was detected as described in the Materials and Methods. The blots were quantified and plotted against La^{3+} incubation time.

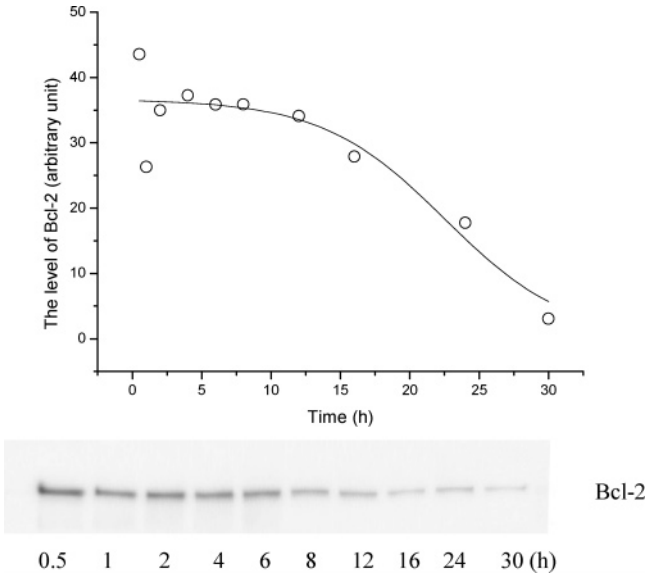


FIGURE 7: Time-dependent decrease of the Bcl-2 protein level upon La^{3+} incubation. Cells were serum-starved for 12 h followed by incubation with 100 μM La^{3+} for the indicated time, and then the cellular Bcl-2 protein was detected as described in the Materials and Methods. The blots were quantified and plotted against La^{3+} incubation time.

because the intracellular chelator BAPTA for both Ca^{2+} and La^{3+} did not affect ERK phosphorylation (Figure 7); in contrast, the protein chelator apoCaM, which binds the metal ions exclusively outside cells, reduced the phosphorylation (*44*). Hence, the binding site of La^{3+} should be at the cell membrane. Using two competitive ligands (malic acid and apoCaM) (Figure 3), the apparent dissociation constant for

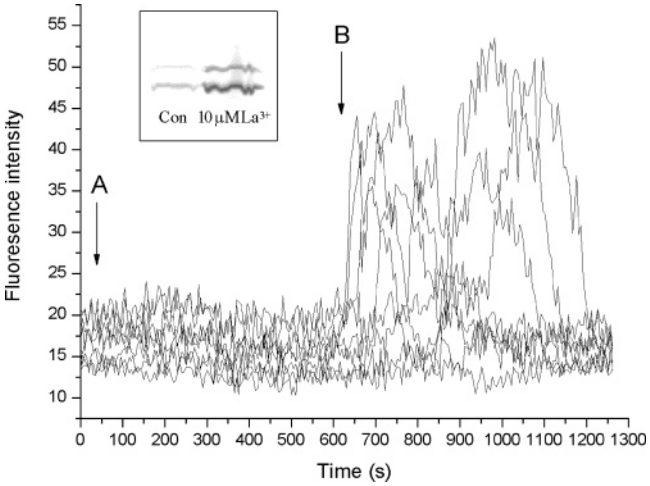


FIGURE 8: Effects of 10 and 100 μM La^{3+} on intracellular Ca^{2+} concentrations monitored by LSCM. Cells were seeded on the collagen-coated Petri dish and loaded with fluo-3-AM for 45 min, and then La^{3+} was added at the indicated time by arrow A (10 μM La^{3+}) and B (100 μM La^{3+}). The time courses of fluorescence in eight representative cells are shown. (Inset) La^{3+} (10 μM) induces ERK phosphorylation detected as described.

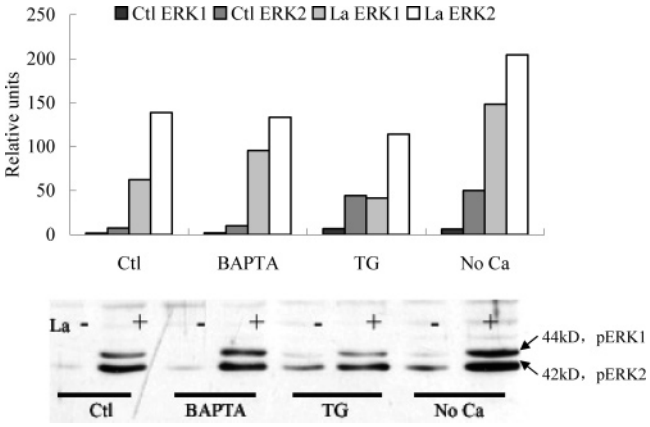


FIGURE 9: Effects of cellular Ca^{2+} on La^{3+}-induced phosphorylation of ERK. The NIH 3T3 cells were preincubated with Ca^{2+}-free buffer (no Ca), intracellular Ca^{2+} pool depletor TG, or intracellular Ca^{2+} chelator BAPTA/AM. Then, the phorphosrylated ERK was blotted, and the blots were quantified as described in the Materials and Methods.

binding of La^{3+} free ions to the putative membrane target was calculated to be about 8 nM. The Hill coefficient (n = 0.86) suggests that there may possibly be only one type of specific binding site for La^{3+} on the putative target.

In recent years, a number of papers indicated that there are CaRs on the plasma membrane of some kinds of cells (*45*), which can be activated by Ca^{2+} or polyvalent cations including Ln ions (*16*) and subsequently induce ERK phosphorylation (*46*). CaR was found to exhibit multiple positive cooperation sites for Ca^{2+}/metal ions, and activation of CaR was also featured by an elevated intracellular Ca^{2+} level. However, NIH 3T3 cells were not reported to express of CaR (*47*), and indeed, no expression of CaR was detected in NIH 3T3 cells by a RT-PCR assay (data not shown). Moreover, at a concentration of 10 μM, La^{3+} was shown to induce ERK phosphorylation but no intracellular Ca^{2+} elevation was observed (Figure 7). Therefore, the CaR pathway can be excluded from La^{3+}-induced phosphorylation of ERK.

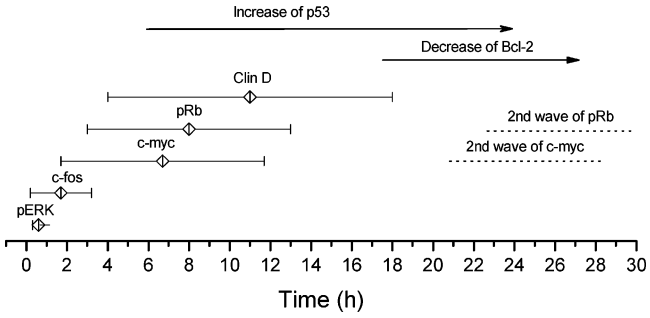
Whether the specific La^{3+}-induced ERK phosphorylation is through a Ca^{2+}-related pathway was examined by the treatment of NIH 3T3 cells with (i) Ca^{2+}-free KRH buffer (removal of extracellular Ca^{2+}), (ii) BAPTA/AM (chelating intracellular free Ca^{2+}), and (iii) TG (exhausting the intracellular Ca^{2+} pool). As shown in Figure 7, all of these treatments did not activate ERK nor did they inhibit La^{3+}-induced ERK phosphorylation, indicating that La^{3+}-induced phosphorylation of ERK could be independent of the Ca^{2+}-signaling pathway. This is in agreement with the previous observation that Ca^{2+} and CaM are not essential to activate ERK2 in cultured fibroblasts in response to various stimuli such as serum; in fact, inhibition of CaM in serum-starved cells induced ERK2 phosphorylation or prolonged activation of ERK2 upon serum addition (*42*). However, depletion of intracellular Ca^{2+} was found to result in the severe loss of cell viability, suggesting that Ca^{2+} is still crucial in cell proliferation of NIH 3T3 cells.

Therefore, all of the evidence suggest that, in NIH 3T3 cell, La^{3+} initiated ERK phosphoylation by binding to an unknown metal-sensing membrane protein, which is a novel metal-sensing mechanism other than CaR.

Nonspecific Activation of ERK upon Incubation with La^{3+}. At high concentrations, La^{3+} also activates ERK in a nonspecific binding manner. There are several possible mechanisms: (i) Elevated intracellular Ca^{2+} might activate ERK. As shown in Figure 7, a high concentration (100 μM) of La^{3+} resulted in a significant increase of intracellular Ca^{2+}. This elevation of intracellular Ca^{2+} should be due to a La^{3+}-inducd Ca^{2+} influx through the L-type calcium channel because both nifedipine (but not flunarizine or TG) and removal of extracellular Ca^{2+} can inhibit it. Hereby, this action of La^{3+} is different from that of La^{3+} above millimolar levels, when La^{3+} is generally recognized as a calcium-channel blocker or antagonist. (ii) Because La^{3+} may permeate the cell membrane at tens of micromolar concentrations, it is possible that La^{3+} might form La-content complexes with CaM, e.g., La_2Ca_2−CaM, in NIH 3T3 cells as proposed previously (*14*, *15*). The hybrid complexes have been shown to bind to CaM-binding proteins but with reduced capacity of activating the targets (*14*, *15*). Hence, the La^{3+}-content CaM might stimulate ERK activation either in a mechanism similar to that described by Bosch et al. (*42*) or (iii) by performing the same actions as in HeLa cells (*44*). We have previously observed that La^{3+} (30 μM or higher) promoted ERK phosphorylation in HeLa cells through a mechanism involving the activation of CaM (*44*), which could be inhibited by a CaM inhibitor, W13.

La^{3+}-Induced ERK Signaling Cascade May Provide a Link between La^{3+}-Induced Cell Proliferation and Apoptosis. The ERK-signaling cascade and related signaling upon incubation with La^{3+} is summarized in Scheme 1 based on the experimental results described above. It was seen that La^{3+} induces a wave of ERK phosphorylation in ~1 h, followed by a wave of c-fos expression (Figures 4 and 5). This is in agreement with a previous postulation (*22*) that phosphorylated ERK would translocate into the nucleus and induce the expression of immediately early genes including *c-fos* and *c-myc*. However, expression of *c-myc* exhibits two waves, suggesting that multiple factors might be involved in triggering *c-myc* expression.

Scheme 1: Sequence of the Signal Transduction in Nih 3T3 Cells upon Incubation with La^{3+}



The expression of cylin D1 was observed a few hours later than the c-fos and c-myc proteins. This is consistent with the postulation that cylin D1 expression is controlled by either ERK (*48*), c-myc (*25*), or c-fos (*22*). Because cylin D1 and c-myc were observed to share the same La^{3+}-concentration dependency (Figure 5b), it is possible that both expressions of c-myc and cyclin D1 may be induced by the same upstream signaling.

Afterward, there was an extended phosphorylation of Rb protein; one wave is peaked at ~8 h, which is earlier than the cyclin D1 expression, and another wave is after the peak of cyclin D1. It has been proposed that cyclin D1 associates with CDK 4/6, sequentially leading to the phosphorylation of the Rb protein (*23*). Therefore, two possible reasons accounting for the first wave of pRb phosphorylation could be (i) from other activators; for instance, the myc induction could cause premature activation of cyclin E-, cyclin A-, and cyclin Dl-associated kinases (*49*), or (ii) an artifact because of the strong and extend cyclin D1 expression induced by La^{3+}.

We have previously shown that the cell proliferation of NIH 3T3 induced by La^{3+} is associated with apoptosis (*18*). Our previous observation included (i) the phosphorylation of ERK that exhibited a high correlation with both the rate of cell proliferation ($r = 0.976$) and degree of cell apoptosis ($r = 0.981$) and (ii) the inhibition of ERK phosphorylation by PD98059 that suppressed both proliferation and apoptosis induced by La^{3+} (*18*). Herein, a high overall correlation ($r = 0.991$) between the expression of cyclin D1/c-myc/p53 and S-phase population was observed, suggesting that the ERK signaling may provide a link for La^{3+}-induced cell proliferation with the apoptosis process.

First, ERK signaling accounts for La^{3+}-induced cell proliferation of NIH 3T3. La^{3+} was observed to induce pRb phosphorylation by cyclin D1-CDK 4/6 activation and/or the c-myc induction. It has been proposed that phosphorylation of pRb will activate transcription factor E2F by dissociation of pRb from Rb−E2F complexes and, in turn, E2F will initiate a set of gene expression driving cells through the G1/S restriction point (*23*). This is consistent with our previous observation that La^{3+} promoted 3T3 cells to pass through the G1/S restriction point and enter S phase (*18*).

Second, ERK signaling is also the pathway leading to apoptosis of NIH 3T3 cells. It was observed that a significant apoptosis of NIH 3T3 cells occurring after 36 h (*18*) followed an increase of the p53 level and a decrease of the Bcl-2 protein level (Scheme 1), suggesting that La^{3+}-induced apoptosis would be p53-related.

It has been reported that sustained activation of c-myc and E2F would result in a p53-dependent cell apoptosis (*36*). One important pathway for cell apoptosis induced by deregulation of c-myc is through the ARF−Mdm2−p53 axis (*24*, *26*−*28*, *31*, *32*). Survival of the cells with deregulated c-myc expression needs a continuous supply of survival factors or additional anti-apoptotic factors, i.e., elevated expression of Bcl-2 (*34*), while overexpression of E2F1 was demonstrated to induce apoptosis under conditions where serum growth factors, which normally impart survival signals, are limiting (*36*).

We observed that (i) La^{3+} resulted in only one pulse of ERK activation; in contrast, serum stimulates two waves of phosphorylation of ERK. Therefore, there could be some missing signals in the La^{3+}-induced proliferating process. (ii) La^{3+} induced the two strong waves of c-myc expression as well as an extended phosphorylation of Rb protein and thus a sustained activation of the E2F-1 transcription factor would be expected. Because Rb/E2F was shown to connect with p53 accumulation (*35*), it is conceivable that La^{3+} induced the elevated level of p53 after the c-myc induction and pRb phosphorylation (Scheme 1). (iii) La^{3+} induced a sharp decrease of Bcl-2 (Figure 7); however, the mechanism for the decrease of Bcl-2 is thus far not clear. These experimental results supported that La^{3+} induced the deregulation of c-myc from ERK signaling and thus resulting in cell apoptosis possibly through the previously proposed ARF−Mdm2−p53 mechanism.

CONCLUSION

The effects of La^{3+} on ERK signaling were investigated to explore the mechanism by which La^{3+} results in cell proliferation associated with cell apoptosis in NIH 3T3 cells. Our data showed that La^{3+} initiated ERK phosphoylation by binding to an unknown metal-sensing membrane protein. The putative protein is sensitive to free La^{3+} ions with a dissociation constant of ∼8 nM. Afterward, activation of *c-fos*, *c-myc*, and *cyclin D1* was in turn observed helping cells to pass through the G1/S restriction point. However, La^{3+}-induced proliferating signaling is not balanced possibly because of the sustained expression of c-myc and phosphorylation of pRb. Subsequently, an increase of the p53 level was observed along with a decrease of the Bcl-2 level. Finally, La^{3+} caused apoptosis of NIH 3T3 cells compatible with a p53-related mechanism.

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