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Short communication

Inhibition of $HgCl_2$-induced mitogen-activated protein kinase activation by LL-Z1640-2 in CCRF-CEM cells

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Abstract

Exposure of $HgCl_2$ to CCRF-CEM human lymphoblastoid cells induced phosphorylation of mitogen-activated protein kinases (MAPKs); extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. LL-Z1640-2, a macrocyclic nonaketide, inhibited $HgCl_2$-induced JNK phosphorylation at 5–100 ng/ml. It also inhibited phosphorylation of ERK and p38 but only at 100 ng/ml. The same doses of radicicol did not suppress MAPKs activation. LL-Z1640-2 (at 100 ng/ml) inhibited $HgCl_2$-induced JNK phosphorylation in NIH 3T3 fibroblasts but not in LLC-PK_1 renal epithelial cells. Thus, LL-Z1640-2 is a potent inhibitor of $HgCl_2$-induced MAPKs activation, especially that of JNK, in CCRF-CEM cells.

Keywords: LL-Z1640-2; Radicicol; Mitogen-activated protein kinases; c-Jun N-terminal kinase; $HgCl_2$

1. Introduction

Mitogen-activated protein kinases (MAPKs) are a family of Ser/Thr protein kinases that transmit extracellular signals into the nucleus (Schaeffer and Weber, 1999). Three subfamilies of MAPKs have been identified; extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) also known as stress-activated protein kinase (SAPK), and p38. These MAPKs require the phosphorylation on one Thr and one Tyr residue for their activation (Robinson and Cobb, 1997).

Radicicol-related macrocyclic nonaketide compounds have been reported to modify MAPK cascades with different specificity (Zhao et al., 1995; Takehana et al., 1999). An anti-fungal antibiotic, radicicol, inhibited ERK activity in untransformed NIH 3T3 cells as well as the *ras* and *mos* oncogenes-transformed cells (Zhao et al., 1995). On the other hand, LL-Z1640-2, a natural compound isolated as an anti-protozoan (Ellestad et al., 1978), has been reported to inhibit anisomycin-induced JNK and p38 activation but not epidermal growth factor-induced ERK activation in Hela cells (Takehana et al., 1999). Interestingly, LL-Z1640-2 did not inhibit tumor necrosis factor-induced JNK activation, suggesting that the inhibition of JNK pathway by LL-Z1640-2 was signal-specific (Takehana et al., 1999). However, mechanisms by which LL-Z1640-2 inhibited JNK activation, and inhibitory effects of LL-Z1640-2 on activation of MAPKs by other extracellular stimuli have not been known.

We have previously found that inorganic mercury, a heavy metal which causes the damage to various organs including renal proximal tubules (Goyer and Cherian, 1995) and immune systems (Lawrence and McCabe, 1995), induced JNK phosphorylation in LLC-PK_1 porcine renal epithelial cells (Matsuoka et al., 2000). Furthermore, exposure to $HgCl_2$ has been reported to induce ERK phosphorylation as well as JNK phosphorylation in murine thymic T lymphocytes (Akhand et al., 1998). We therefore examined whether LL-Z1640-2 can inhibit phosphorylation of MAPKs induced by $HgCl_2$ exposure in CCRF-CEM human T lymphoblastoid, LLC-PK_1 and NIH 3T3 cells.

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2. Materials and methods

CCRF-CEM cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Exponentially growing CCRF-CEM cells were plated at 3×10^6 cells/well in 6-well culture plates and subsequently used for the experiments. LLC-PK_1 cells were grown in medium 199 supplemented with 5% heat-inactivated fetal bovine serum, and NIH 3T3 cells were in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. LLC-PK_1 and NIH 3T3 cells were plated at 5×10^5 cells/well in 6-well culture plates and cultured for 1 day prior to the experiments.

$HgCl_2$ was dissolved in water and sterilized by filtration. The stock solution (10 mg/ml) of LL-Z1640-2 (provided by Pharmaceutical Research Laboratories, Ajinomoto, Kawasaki, Japan) and radicicol (Calbiochem, La Jolla, CA, USA) were dissolved in dimethyl sulfoxide (DMSO). Cells were preincubated with serum-free medium containing either 0.001% DMSO, LL-Z1640-2 or radicicol (10, 50 or 100 ng/ml) for 30 min, and then incubated with or without 20 μM $HgCl_2$ for 1 h at 37°C.

After the incubation with $HgCl_2$, cells were washed with phosphate-buffered saline and lysed with sodium dodecyl sulfate-polyacrylamide gel Laemmli sample buffer. The levels of phosphorylated ERK, total ERK, phosphorylated JNK, total JNK, phosphorylated p38, and total p38 were determined by Western blot analysis as described previously (Matsuoka and Igisu, 1998). The antibodies used were phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody, phosphorylation state-independent p44/42 MAPK antibody, phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) antibody, phosphorylation state-independent SAPK/JNK antibody, phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibody, and phosphorylation state-independent p38 MAPK antibody (all from New England Biolabs, Beverly, MA, USA). The bands on the developed film were quantified with NIH Image Version 1.61.

Results were expressed as the mean ± S.E.M. The statistical significance was determined by one-way analysis of variance followed by the Bonferroni multiple comparison test. $P < 0.05$ was considered as statistically significant.

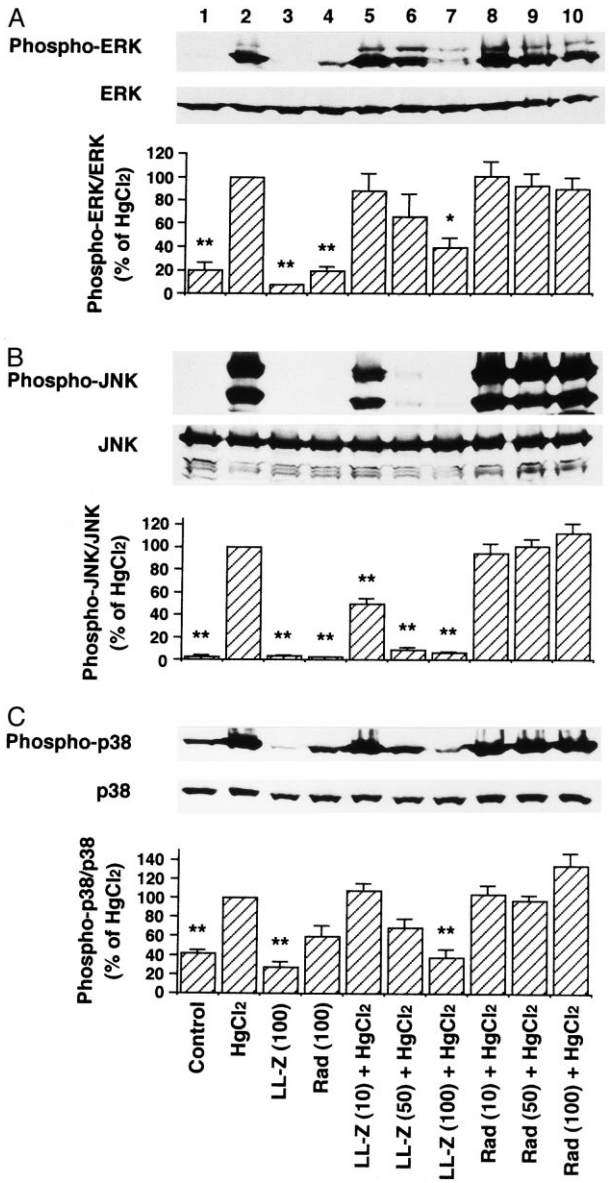

Fig. 1. Effects of LL-Z1640-2 (LL-Z) and radicicol (Rad) on $HgCl_2$-induced phosphorylation of ERK (A), JNK (B) and p38 (C). CCRF-CEM cells were preincubated with LL-Z (10, 50 and 100 ng/ml) or Rad (10, 50 and 100 ng/ml) for 30 min, and then incubated with or without 20 μM $HgCl_2$ for 1 h. Cell lysates were subjected to Western immunoblotting. Based on the densitometric analyses, the levels of phosphorylated ERK (A), JNK (B) and p38 (C) were determined. Each value was expressed as the ratio of phosphorylated MAPK level to its corresponding total MAPK level, and the value of $HgCl_2$ (0.001% DMSO plus 20 μM $HgCl_2$) was set to 100%. Each column and bar represents the mean ± S.E.M. of four experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to $HgCl_2$.

3. Results

Incubation with 20 μM $HgCl_2$ for 1 h elevated the levels of phosphorylated form of ERK, JNK and p38 markedly in CCRF-CEM cells while those of total (phosphorylation state-independent) ERK, JNK and p38 were not changed (Fig. 1A,B,C, lane 2). Treatment with 100 ng/ml of LL-Z1640-2 or radicicol alone did not affect the levels of phosphorylated and total MAPKs (Fig. 1A,B,C, lanes 3 and 4). $HgCl_2$-induced elevation of phosphorylated JNK level was decreased by the treatment with LL-Z1640-2 in a dose-dependent manner. The level of phosphorylated JNK in cells treated with 1, 5, 10, 25, 50 and 100 ng/ml LL-Z1640-2 was found to be 95.0 ± 4.0 ($n = 3$, $P > 0.05$), 76.2 ± 6.8 ($n = 3$, $P < 0.01$), 49.7 ± 4.7 ($n = 4$, $P < 0.01$,

Fig. 1B, lane 5), 37.8 ± 4.5 ($n = 3$, $P < 0.01$), 9.0 ± 2.2 ($n = 4$, $P < 0.01$, Fig. 1B, lane 6) and 6.0 ± 1.4% ($n = 4$, $P < 0.01$, Fig. 1B, lane 7) of that of cells treated with $HgCl_2$ only, respectively. While $HgCl_2$-induced elevations of phosphorylated ERK and p38 levels were also decreased by the treatment with LL-Z1640-2 depending on its concentration (Fig. 1A,C, lanes 5, 6 and 7), the statistically significant reduction of ERK and p38 phosphorylation was found only with 100 ng/ml LL-Z1640-2 ($P < 0.05$ and $P < 0.01$, respectively, Fig. 1A,C, lane 7). In contrast to LL-Z1640-2, treatment with 10, 50 or 100 ng/ml radicicol did not suppress $HgCl_2$-induced phosphorylation of ERK, JNK or p38 ($P > 0.05$, Fig. 1A,B,C, lanes 8, 9 and 10).

Incubation with 20 μM $HgCl_2$ for 1 h induced the significant accumulation of phosphorylated JNK in both LLC-PK_1 cells (Fig. 2, lane 2) and NIH 3T3 cells (Fig. 2, lane 6). Inhibitory effects of LL-Z1640-2 (at 100 ng/ml) on $HgCl_2$-induced JNK phosphorylation was observed in NIH 3T3 cells ($P < 0.001$, Fig. 2, lane 8) but not in LLC-PK_1 cells ($P > 0.05$, Fig. 2, lane 4). The levels of total JNK were not affected by the treatment with $HgCl_2$, LL-Z1640-2 or both in LLC-PK_1 cells (Fig. 2, lanes 2, 3 and 4) and in NIH 3T3 cells (Fig. 2, lanes 6, 7 and 8).

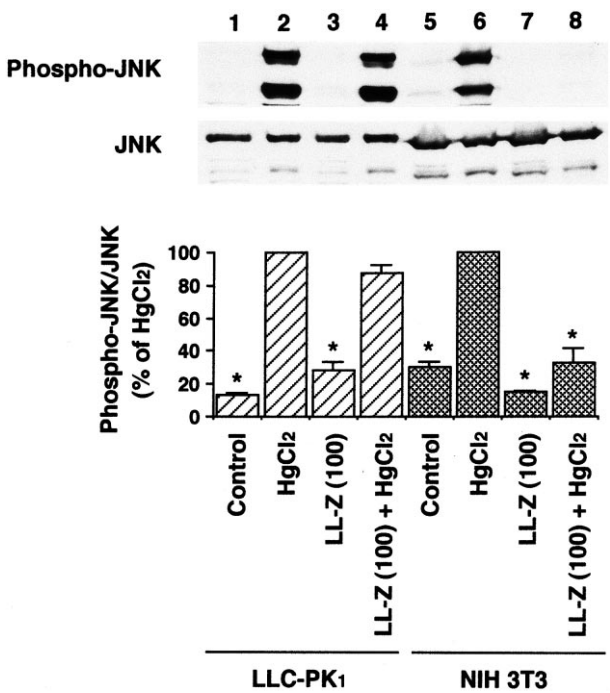

Fig. 2. Effects of LL-Z1640-2 (LL-Z) on $HgCl_2$-induced JNK phosphorylation in LLC-PK_1 and NIH 3T3 cells. LLC-PK_1 and NIH 3T3 cells were preincubated with or without 100 ng/ml LL-Z for 30 min, and then incubated with or without 20 μM $HgCl_2$ for 1 h. Cell lysates were subjected to Western immunoblotting. Based on the densitometric analyses, the level of phosphorylated JNK was determined. Each value was expressed as the ratio of phosphorylated JNK level to total JNK level, and the value of $HgCl_2$ (0.001% DMSO plus 20 μM $HgCl_2$) in each cell line was set to 100%. Each column and bar represents the mean ± S.E.M. of three experiments. * $P < 0.001$ compared to $HgCl_2$ in each cell line.

4. Discussion

A macrocyclic nonaketide compound, LL-Z1640-2, has been reported to inhibit anisomycin-induced JNK and p38 activation in Hela cells (Takehana et al., 1999). In the present study using $HgCl_2$, MAPKs including ERK, JNK and p38 were phosphorylated in CCRF-CEM cells, and treatment with LL-Z1640-2 suppressed their phosphorylation without affecting the total protein levels of each MAPK. While the most marked inhibitory effects of LL-Z1640-2 (at 10 ng/ml) was observed on JNK, phosphorylation of ERK and p38 was also inhibited concomitantly at a higher concentration (100 ng/ml). These results indicate that the effect of LL-Z1640-2 was not specific to JNK pathway, and might act on the upstream signal transduction pathways responsible for the activation of three MAPK family members in response to $HgCl_2$ exposure.

While LL-Z1640-2 and radicicol are chemically related compounds, their effects on MAPKs were shown to be different. In contrast to LL-Z1640-2, treatment with radicicol did not inhibit $HgCl_2$-induced phosphorylation of ERK, JNK or p38 even when a higher concentration (100 ng/ml) was used. Consistent with this finding, radicicol did not reduce JNK and p38 activities in Hela cells treated with anisomycin (Takehana et al., 1999). It has been reported that $HgCl_2$ activated Src family of nonreceptor protein tyrosine kinases such as $p56^{lck}$ in murine thymocytes (Nakashima et al., 1994) and $p60^{c\text{-}src}$ in NIH 3T3 cells (Pu et al., 1996). On the other hand, radicicol could inhibit Src proteins such as $p60^{v\text{-}src}$ (Kwon et al., 1992), $p60^{c\text{-}src}$ and $p53/56^{lyn}$ (Chanmugam et al., 1995), and also downstream Raf kinase (Soga et al., 1998). However, treatment with neither LL-Z1640-2 nor radicicol (at 100 ng/ml for 30 min) suppressed significantly the phosphorylation of $p56^{lck}$ in CCRF-CEM cells exposed to 20 μM $HgCl_2$ for 2 min (data not shown), suggesting that Src might not be responsible for the different effects of macrocyclic nonaketide compounds on MAPKs phosphorylation.

In cell types other than CCRF-CEM cells, inhibitory effect of LL-Z1640-2 on $HgCl_2$-induced JNK phosphorylation was observed in NIH 3T3 fibroblasts but not in LLC-PK_1 renal epithelial cells, one of the target tissues of mercury (Goyer and Cherian, 1995; Duncan-Achanzar et al., 1996). While the precise mechanisms underlying the lack of inhibition of JNK activation in LLC-PK_1 cells are not known, uptake of LL-Z1640-2 into cells, its intracellular metabolism, or pathway leading to JNK activation by $HgCl_2$ exposure might be different among cell types.

In summary, while both LL-Z1640-2 and radicicol are macrocyclic nonaketide compounds, their effects on the activation of MAPKs in CCRF-CEM cells exposed to $HgCl_2$ were found to be different. LL-Z1640-2 was a potent inhibitor of $HgCl_2$-induced MAPKs activation, especially that of JNK, in the cell type-dependent manner. Treatment with radicicol failed to inhibit MAPKs activation by $HgCl_2$ exposure. The mechanism by which LL-

Z1640-2 inhibits stress-induced activation of MAPK cascades and the possible immunomodulatory effects of LL-Z1640-2 await further investigations.

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