Heavy metal chelator TPEN attenuates fura-2 fluorescence changes induced by cadmium, mercury and methylmercury

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ABSTRACT. Stimulation with heavy metals is known to induce calcium (Ca \(^{2+}\)) mobilization in many cell types. Interference with the measurement of intracellular Ca\(^{2+}\) concentration by the heavy metals in cells loaded with Ca\(^{2+}\) indicator fura-2 is an ongoing problem. In this study, we analyzed the effect of heavy metals on the fura-2 fluorescence ratio in human SH-SY5Y neuroblastoma cells by using TPEN, a specific cell-permeable heavy metal chelator. Manganese chloride (30–300 \(\mu\)M) did not cause significant changes in the fura-2 fluorescence ratio. A high concentration (300 \(\mu\)M) of lead acetate induced a slight elevation in the fura-2 fluorescence ratio. In contrast, stimulation with cadmium chloride, mercury chloride or MeHg (3–30 \(\mu\)M) elicited an apparent elevation of the fura-2 fluorescence ratio in a dose-dependent manner. In cells stimulated with 10 or 30 \(\mu\)M cadmium chloride, the addition of TPEN decreased the elevated fura-2 fluorescence ratio to basal levels. In cells stimulated with mercury or MeHg, the addition of TPEN significantly decreased the elevation of the fura-2 fluorescence ratio induced by lower concentrations (10 \(\mu\)M) of mercury or MeHg, but not by higher concentrations (30 \(\mu\)M). Pretreatment with Ca\(^{2+}\) channel blockers, such as verapamil, 2-APB or lanthanum chloride, resulted in different effects on the fura-2 fluorescence ratio. Our study provides a characterization of the effects of several heavy metals on the mobilization of divalent cations and the toxicity of heavy metals to neuronal cells.

KEYWORDS: Fura-2, heavy metal, SH-SY5Y, TPEN

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Heavy metals are distributed as environmental pollutants, and human and animal exposure to excessive levels of heavy metals is a global public health problem. Although some heavy metals, such as copper (Cu\(^{2+}\)), zinc (Zn\(^{2+}\)), manganese (Mn\(^{2+}\)) and iron (Fe\(^{2+}\)), are essential for maintaining normal gene transcription, cell motility, exocytosis, cell growth anding molecule that regulates many cellular processes, such as intracellular calcium concentrations ([Ca\(^{2+}\)]\(_{i}\)) in neuronal stimulation with heavy metals can induce an increase in mercury in cortical neurons [36], by manganese in cerebellar granule neurons [25], by cadmium chloride, mercury chloride or MeHg (3–30 \(\mu\)M) elicited an apparent elevation of the fura-2 fluorescence ratio in a dose-dependent manner. In cells stimulated with 10 or 30 \(\mu\)M cadmium chloride, the addition of TPEN decreased the elevated fura-2 fluorescence ratio to basal levels. In cells stimulated with mercury or MeHg, the addition of TPEN significantly decreased the elevation of the fura-2 fluorescence ratio induced by lower concentrations (10 \(\mu\)M) of mercury or MeHg, but not by higher concentrations (30 \(\mu\)M). Pretreatment with Ca\(^{2+}\) channel blockers, such as verapamil, 2-APB or lanthanum chloride, resulted in different effects on the fura-2 fluorescence ratio. Our study provides a characterization of the effects of several heavy metals on the mobilization of divalent cations and the toxicity of heavy metals to neuronal cells.

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and lanthanum chloride, on changes in the fura-2 fluorescence ratio induced by heavy metals.

**MATERIALS AND METHODS**

**Cell culture:** Human neuroblastoma cell line SH-SY5Y (ATCC, Manassas, VA, U.S.A.), were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) mixed 1:1 with Ham’s F-12 nutrient mixture (Sigma-Aldrich, St. Louis, MO, U.S.A.) containing 10% fetal bovine serum, 100 unit/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Two days before experimentation, cells were seeded at a density of 7 × 10⁴ cells/cm² in a 96-well plate.

**Measurement of fura-2 fluorescence changes:** Cells in a 96-well plate were serum-starved for 4 hr; calcium indicator fura-2 was then loaded into the cells by using Calcium kit II fura-2 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. In brief, SH-SY5Y cells were incubated with 5 µM fura-2/AM in the presence of 0.04% Pluronic F-127, a dispersing agent to improve the efficiency of loading with fura-2, and 1.25 mM probenecid, a blocker of organic anion transport to prevent leakage of fura-2 from cells [8]. After 1 hr incubation at 37°C, fura-2 fluorescence was measured at 500 nm emission after excitation at 340 nm (F340) or 380 nm (F380) using an Infinite M200 plate reader (Tecan, Männedorf, Switzerland) at 37°C. The change in [Ca²⁺], was reflected by the ratio of F340 and F380. To determine the changes in fura-2 fluorescence ratio induced by heavy metal compounds, cells were treated with manganese chloride (Sigma-Aldrich), lead acetate (Wako, Osaka, Japan), cadmium chloride (Kanto Chemical, Tokyo, Japan), mercuric chloride (Wako) and MeHg chloride (Kanto Chemical) dissolved in distilled water. We confirmed that the cells adhered to the bottom of the plate after 6 hr exposure to heavy metal compounds. The cells were also treated with three Ca²⁺ channel blockers, lanthanum chloride (Wako) dissolved in distilled water, verapamil (Sigma-Aldrich) and 2-APB dissolved in DMSO, 30 min before heavy metal exposure. The heavy metal chelator TPEN was dissolved in DMSO and added 3 hr after the stimulation with heavy metals to determine the contribution of endogenous and exogenous heavy metals on fura-2 fluorescence changes. We measured the effect of TPEN (20 µM) on the fura-2 fluorescence ratio after a 10 min treatment with TPEN, since our preliminary experiments showed that the effect of TPEN on fura-2 fluorescence reached maximum and stabilized within 10 min of the treatment.

**Data analysis:** Results are expressed as the mean ±SEM. Differences between means were evaluated by Student’s t-test or Bonferroni’s correction for multiple comparisons; P values<0.05 were considered significant.

**RESULTS**

Fura-2 fluorescence changes induced by heavy metals: Figure 1 shows the changes in the fura-2 fluorescence ratio in SH-SY5Y cells by stimulation with manganese chloride, lead acetate, cadmium chloride, mercury chloride or MeHg. In cells without heavy metal stimulation (controls), we did not observe significant changes in the fura-2 fluorescence ratio within 6 hr under experimental conditions (data not shown). First, we examined the effect of manganese chloride and lead acetate on fura-2 fluorescence, since these metals are well known to be neurotoxic substances and have been shown to stimulate [Ca²⁺] [31, 37]. Manganese chloride (30–300 µM) did not cause a significant change in the fura-2 fluorescence ratio within 6 hr of exposure (Fig. 1A). Although manganese chloride had no effect on the fura-2 fluorescence ratio, we observed that it induced a quenching of fluorescent signals at both F340 and F380 in a dose-dependent manner (data not shown). Lead (30–100 µM) did not cause changes in the fura-2 fluorescence ratio (Fig. 1B). However, a higher concentration (300 µM) of lead induced a slight but significant elevation in the fura-2 fluorescence ratio. In contrast, stimulation with cadmium chloride, mercury chloride or MeHg elicited an apparent elevation of the fura-2 fluorescence ratio in a dose-dependent manner (Fig. 1C, 1D and 1E). The peak increase in the fura-2 fluorescence ratio from basal level (ΔRatio (340/380)) was induced by 30 µM cadmium chloride (2.9 ± 0.1 at 6 hr), 30 µM mercury chloride (2.3 ± 0.4 at 3 hr) and 30 µM MeHg (3.3 ± 0.1 at 4 hr). We confirmed that Ca²⁺ ionophore ionomycin (3 µM) induced significant increase in the ΔRatio (340/380) (0.5 ± 0.1 at 3 min).

**Effect of TPEN on fura-2 fluorescence changes induced by cadmium chloride, mercury chloride or MeHg:** Since we observed an apparent elevation of the fura-2 fluorescence ratio induced by stimulation with cadmium chloride, mercury chloride and MeHg in SH-SY5Y cells, we examined the contribution of heavy metal cations to the increase in the fura-2 fluorescence ratio by using TPEN, a cell-permeable chelator for heavy metal cations with a low affinity for Ca²⁺ [1]. In cells stimulated with 10 or 30 µM cadmium chloride, the addition of TPEN at 3 hr after exposure significantly decreased the elevated fura-2 fluorescence ratio to the basal levels within 10 min (119.6 ± 2.4% or 109.0 ± 1.5% decrease in ΔRatio (340/380) by 5, 10 or 20 µM TPEN, respectively, n=4). In mercury chloride or MeHg treated cells, the addition of TPEN significantly decreased the increase in the fura-2 fluorescence ratio was dependent on an increase in intracellular heavy metal cations but not intracellular Ca²⁺ (Fig. 2A). We observed that the effect of TPEN on the elevated fura-2 fluorescence ratio induced by 30 µM cadmium chloride was dose-dependent (2.4 ± 3.4%, 69.3 ± 3.5% or 98.3 ± 0.5% decrease in ΔRatio (F340/F380) by 5, 10 or 20 µM TPEN, respectively, n=4). In mercury chloride or MeHg treated cells, the addition of TPEN significantly decreased the elevation of ΔRatio (F340/F380) induced by a lower concentration (10 µM) of mercury chloride (173.7 ± 19.4% decrease in ΔRatio (F340/F380)) or MeHg (110.7 ± 11.1% decrease in ΔRatio (F340/F380)) (Fig. 2B and 2C). However, although the addition of TPEN partially decreased the elevation of ΔRatio (F340/F380) induced by a higher concentration (30 µM) of mercury chloride (44.1 ± 18.0% decrease in ΔRatio (F340/F380)) or MeHg (34.4 ± 15.1% decrease in ΔRatio (F340/F380)), the effect was not significant.

**Effect of Ca²⁺ channel blockers on fura-2 fluorescence...**
changes induced by cadmium chloride, mercury chloride or MeHg: Finally, we analyzed the effect of pretreatment with Ca\(^{2+}\) channel blockers on the increase in the fura-2 fluorescence ratio induced by cadmium chloride, mercury chloride or MeHg. Pretreatment with verapamil, an L-type Ca\(^{2+}\) channel blocker [24], or lanthanum chloride, a non-specific cation channel blocker, significantly inhibited the fura-2 fluorescence ratio induced by cadmium chloride (Fig. 3). In contrast, 2-APB, an inositol 1,4,5-trisphosphate (IP\(_3\)) receptor and canonical transient receptor potential cation (TRPC) channel blocker [27], caused only a slight inhibition of the cadmium chloride-induced increase in the fura-2 fluorescence ratio. The fura-2 fluorescence ratio induced by mercury chloride was not affected by treatment with verapamil, 2-APB or lanthanum chloride (Fig. 4). Although verapamil and lanthanum chloride did not cause significant effects, 2-APB significantly suppressed the increase in the fura-2 fluorescence ratio induced by MeHg (Fig. 5).

DISCUSSION

Fura-2 is a UV-excited ratiometric indicator dye for mea-
suring \([\text{Ca}^{2+}]\); [12]. Upon binding of fura-2 to \(\text{Ca}^{2+}\), \(\text{Cd}^{2+}\), \(\text{Pb}^{2+}\) and \(\text{Zn}^{2+}\), the emission fluorescence intensity increases at 340 nm (F340) and decreases at 380 nm (F380) for the unbound form [2, 17, 33]. This change in fluorescence intensity results in elevation of the fura-2 fluorescence ratio (F340/F380). In contrast, the binding of fura-2 to some heavy metals, such as \(\text{Mn}^{2+}\) and \(\text{Fe}^{2+}\) [16], quenches the fluorescence (both at F340 and F380) [12, 19, 21]. In this study, we found that manganese chloride did not cause significant changes in the fura-2 fluorescence ratio in SH-SY5Y cells. However, we observed a quenching of the fluorescent signal at both F340 and F380. Thus, it is likely that the \(\text{Mn}^{2+}\) penetrates cells and quenches the fura-2 fluorescence.

Lead acetate caused a significant increase in the fura-2 fluorescence ratio only at a higher concentration (300 \(\mu\)M). Sukumar and Beech (2010) reported that stimulation with low lead acetate concentrations induced an increase in \([\text{Ca}^{2+}]\) through the TRPC5 channel in fura-2-loaded HEK 293 cells with TRPC5 overexpression [29]. Since expression of the TRPC5 channel in SH-SY5Y cells has been suggested [5], TRPC5 might mediated the increase in \([\text{Ca}^{2+}]\) by a higher concentration of lead acetate, while it may not have been enough to induce an apparent increase in the fura-2 fluorescence ratio at lower concentrations.

We found that cadmium chloride, mercury chloride and MeHg caused an apparent increase in the fura-2 fluorescence ratio in a dose-dependent manner. Therefore, we analyzed the contribution of endogenous or exogenous metal cations to the fura-2 fluorescence change using TPEN, a cell-permeable chelator for heavy metals. The increase in the fura-2 fluorescence ratio by stimulation with 10 or 30 \(\mu\)M cadmium chloride was inhibited to the basal level by the addition of TPEN, suggesting that the increase in the fura-2 fluorescence ratio was dependent on heavy metal cations but not intracellular \(\text{Ca}^{2+}\). Given that \(\text{Ca}^{2+}\) channels mediate \(\text{Cd}^{2+}\) influx in many cell types [6], it is likely that the change in the fura-2 fluorescence ratio induced by cadmium chloride is caused by an influx of \(\text{Cd}^{2+}\), which binds fura-2 with high affinity and activates its spectral responses, as \(\text{Ca}^{2+}\) does [17]. In support of this explanation, we observed that verapamil and lanthanum chloride partially inhibited the increase in the fura-2 fluorescence ratio induced by cadmium chloride. Furthermore, Hinkle et al. (1987) reported that verapamil reduced cell death induced by cadmium chloride in a pituitary cell line [16]. On the other hand, cellular uptake of \(\text{Cd}^{2+}\) by metal transporters, such as divalent metal transporter 1 (DMT1), Zrt/Irt-related protein (ZIP) 8 and ZIP14, had been reported [32]. Although DMT1 and ZIP14 are expressed in SH-SY5Y cells [11, 34], the contribution to the \(\text{Cd}^{2+}\) uptake has not been known. The involvement of these metal transporters in the cadmium-induced increase in fura-2 fluorescence ratio needs to be clarified in future studies.

The addition of TPEN to cells exposed to a low concentration of mercury chloride or MeHg (10 \(\mu\)M) returned the increased fura-2 fluorescence ratio to basal level, while a higher concentration (30 \(\mu\)M) of mercury chloride or MeHg caused an increase in the fura-2 fluorescence ratio that was only partially sensitive to the TPEN treatment. These results suggest that higher concentrations of mercury chloride and MeHg induce an increase in the fura-2 fluorescence ratio through an increase in intracellular \(\text{Ca}^{2+}\) in SH-SY5Y cells, in addition to the increase in intracellular metal cation levels. Since \(\text{Hg}^{2+}\) and MeHg do not cause perturbation of fura-2 fluorescence [15, 21], the increase in the fura-2 fluorescence ratio which was sensitive to treatment with TPEN may be dependent on endogenous metal cation mobilization. A contribution by endogenous \(\text{Zn}^{2+}\) mobilization in measurement of \([\text{Ca}^{2+}]\), by fura-2 has been proposed [22], and, in fact,

![Fig. 2. Effect of TPEN on fura-2 fluorescence changes induced by stimulation with cadmium chloride, mercury chloride or MeHg. Contribution of heavy metal cations to the increase in the fura-2 fluorescence ratio was estimated by addition of TPEN (20 \(\mu\)M) 3 hr after stimulation with 10 or 30 \(\mu\)M cadmium chloride (A), mercury chloride (B) or MeHg (C) by measurement of \(\Delta\text{Ratio (F340/F380)}\) before and after the addition of TPEN. The values are expressed as mean ±SEM (n=8). *P<0.05 vs. \(\Delta\text{Ratio (F340/F380)}\) before the addition of TPEN.](image-url)
Elevation of [Zn²⁺], by MeHg was reported in synaptosomes [7]. Taken together, it is likely that the increase in [Zn²⁺], at least in part, contributes to a TPEN-sensitive increase in the fura-2 fluorescence ratio induced by exogenous heavy metals. In contrast with cadmium chloride, a MeHg-induced increase in the fura-2 fluorescence ratio was suppressed by 2-APB but not by verapamil or lanthanum chloride, although alteration of MeHg-induced Ca²⁺ mobilization by treatment with nifedipine, an L-type Ca²⁺ channel blocker, has been reported in cerebellar granule cells [23], a neuronal cell line [13, 14, 23] and spinal motor neurons [28]. In agreement with previous reports that MeHg induced an increase in inositol phosphate levels and activated TRPC channels [14, 30, 38], 2-APB, an IP₃ receptor and TRPC channel blocker [27], inhibited an increase in the fura-2 fluorescence ratio induced by MeHg. In this study, significant inhibitory effects were not
observed in mercury chloride-induced increases in the fura-2 fluorescence ratio after treatment with verapamil, 2-APB or lanthanum chloride. Recently, Xu et al. (2012) reported that a mercury-induced increase in $[\text{Ca}^{2+}]_i$ was inhibited by MK801, an antagonist of the NMDA receptor, in cultured cortical neurons [36]. Expression of the NMDA receptor in SH-SY5Y cells has also been reported [3]. The involvement of NMDA receptors or other types of $\text{Ca}^{2+}$ channels, with the mobilization of $[\text{Ca}^{2+}]_i$, by heavy metals in SH-SY5Y cells remains to be clarified.

In this study, we showed that the elevation of the fura-2 fluorescence ratio is dependent not only on $\text{Ca}^{2+}$ but also on heavy metal cations in SH-SY5Y cells. Furthermore, the contribution of heavy metal cations to the changes in the fura-2 fluorescence ratio induced by exogenous heavy metals was metal- and concentration-specific. Our study provides a characterization of the effects of several heavy metals on the mobilization of divalent cations and the toxicity of heavy metals to neuronal cells.

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