Arch Toxicol (2012) 86:1667–1680
DOI 10.1007/s00204-012-0878-3

INORGANIC COMPOUNDS

Endosomes and lysosomes are involved in early steps of Tl(III)-mediated apoptosis in rat pheochromocytoma (PC12) cells

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Received: 22 February 2012 / Accepted: 23 May 2012 / Published online: 6 June 2012

Abstract The mechanisms that mediate thallium (Tl) toxicity are still not completely understood. The exposure of rat pheochromocytoma (PC12) cells to Tl(I) or Tl(III) activates both mitochondrial (Tl(I) and Tl(III)) and extrinsic (Tl(III)) pathways of apoptosis. In this work we evaluated the hypothesis that the effects of Tl(III) may be mediated by the damage to lysosomes, where it might be incorporated following the route of iron uptake. PC12 cells exposed for 3 h to 100 μM Tl(III) presented marked endosomal acidification, effect that was absent when cells were incubated in a serum-free medium and that was fully recovered when the latter was supplemented with transferrin. After 6 h of incubation the colocalization of cathepsins D and B with the lysosomal marker Lamp-1 was decreased together with an increase in the total activity of the enzymes. A permanent damage to lysosomes after 18 h of exposure was evidenced from the impairment of acridine orange uptake. Cathepsin D caused the cleavage of pro-apoptotic protein BID that is involved in the activation of the intrinsic pathway of apoptosis. Supporting that, BID cleavage and the activation of caspase 3 by Tl(III) were fully prevented when cells were preincubated with cathepsin D inhibitor (pepstatin A) and only partially prevented when cathepsin B inhibitor (E64d) was used. None of these inhibitors affected BID cleavage or caspase 3 activation in Tl(I)-treated cells. Together, experimental results support the role of Tl(III) uptake by the acidic cell compartments and their involvement in the early steps of Tl(III)-mediated PC12 cells apoptosis.

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Keywords Thallium · Endosomes · Lysosomes · Acidic compartments · Apoptosis · Cathepsin · Caspase · Transferrin · Toxicity

Abbreviations

Ac-DEVD-pNA	N-acetyl-Asp-Glu-Val-Asp p-nitroanilide
AIF	Apoptosis-inducing factor
Apaf-1	Apoptosis protease-activating factor 1
Bax	Bcl-2-associated X protein
BID	BH3·interacting domain death agonist
DHE	Dihydroethidine
DMEM	Dulbecco′s modified Eagle's medium
DTT	Dithiotreitol
E64d	2S,3S-trans-(ethoxycarbonyloxirane-2-carbonyl)-L-leucine-(3-methylbutyl) amide
Endo G	Endonuclease G
I.O.D.	Integrated optical density
Lamp1	Lysosomal membrane protein 1
MDC	Monodansylcadaverine
PBS	Phosphate buffer saline
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene fluoride
Tf	Transferrin
TCA	Trichloroacetic acid
Z-RR-pNA	Z-Arg-Arg p-nitroanilide

Introduction

The heavy metal thallium (Tl) is a normal component of the earth's crust. However, since this metal forms salts and minerals, its bioavailability is almost negligible. The mobilization of Tl during mining together with its use in certain

industries causes an increase in Tl concentration in the air, water, and soils (ATSDR 1999). Therefore, it becomes available to animals and humans through its incorporation into the food chain (Bunzl et al. 2001; Heim et al. 2002; Lin et al. 2001). Human intoxication with Tl occurs by oral, dermal, or respiratory routes (Peter and Viraraghavan 2005; Repetto et al. 1998). The deposition of this metal in tissues affects several systems, including central and peripheral nervous system where it causes demyelination and neurodegeneration (Douglas et al. 1990; Galvan-Arzate and Santamaria 1998; Heim et al. 2002; Moore et al. 1993). Along with lead, cadmium, and mercury, Tl is considered as a priority pollutant by the US Environmental Protection Agency (http://water.epa.gov).

This metal has two oxidation states, the monovalent (Tl(I)) and trivalent (Tl(III)) cations, being the latter a strong oxidant (Tl(III)/Tl(I) ε^0: +1.25 mV). Clinical symptoms of human intoxication with Tl(I) are well described (Schaub 1996); however, the molecular mechanisms that mediate its toxicity, along with those of Tl(III), are still poorly understood. Our group has demonstrated that after the exposure to Tl(I) or Tl(III) (10–100 μM), the viability of rat pheochromocytoma (PC12) cells is significantly decreased (Hanzel and Verstraeten 2006). These cations cause mitochondrial depolarization and the increase in H_2O_2 content, stressing the relevance of mitochondria as a target of Tl toxicity (Hanzel and Verstraeten 2006; Pourahmad et al. 2010). Supporting that, we have demonstrated that in these cells, Tl(I) and Tl(III) induce apoptosis through the activation of the intrinsic (mitochondrial) pathway (Hanzel and Verstraeten 2009). Interestingly, Tl(III) but not Tl(I) activates the extrinsic pathway of apoptosis as well, an event that appears early in Tl(III) exposure (Hanzel and Verstraeten 2009). Collectively, experimental evidence indicates that Tl(I) and Tl(III) exert their toxic effects through the activation of different pathways. Also, they allow us to hypothesize that Tl(III) not necessarily must be reduced to Tl(I) in the extracellular milieu to enter the cells, but it can possibly be incorporated under its trivalent form. Since Tl(III) binds in vitro to transferrin (Harris and Messori 2002), it may reach cells following the route of Fe uptake. If true, Tl(III) would accumulate within late endosomes and promote their destabilization, leakage, and the release of hydrolytic enzymes, such as cathepsins, able to trigger lysosome-dependent apoptosis. This hypothesis and its relationship with our previous results were investigated in the current work.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM high glucose) was purchased from Gibco BRL (Grand Island, NY, USA); donor horse serum was from PAA Laboratories GmbH (Pasching, Germany); fetal bovine serum was from Natocor (Córdoba, Argentina); thallium(I) nitrate was from Fluka (Milwaukee, WI, USA); and thallium(III) nitrate was from Alfa Æsar (Ward Hill, MA, USA). Acridine orange, monodansylcadaverine (MDC), human apo-transferrin, bovine hemoglobin, pepstatin A, dihydroethidine (DHE), Hoechst 32258, and all the other reagents had the highest quality available and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary polyclonal antibodies against cathepsin D, cathepsin B, full-length BID (BH3 interacting domain death agonist), full-length and cleaved BID, Lamp-1 (lysosomal-associated membrane protein 1), horseradish peroxidase-conjugated anti-mouse, FITC-conjugated anti-rabbit, and Texas Red-conjugated anti-goat antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence system (ECL plus) for Western immunoblot was purchased from GE Healthcare (Arlington Heights, IL, USA); protease inhibitor cocktail was from Roche Diagnostics GmbH (Mannheim, Germany); PVDF membranes were from Bio-Rad Corp. (Hercules, CA, USA); caspase 3 substrate (Ac-DEVD-pNA), cathepsin B substrate (Z-RR-pNA), and E64d were from Biomol (Plymouth, PA, USA); eighteen-millimeter circle cover slips were from Ted Pella Inc. (Redding, CA, USA); sterile fluorescence apt 96-well plates were from Porvair Science Ltd. (Leatherhead, United Kingdom); and Prolong® Gold antifade reagent was from Life Technologies (Grand Island, NY, USA).

Tl solutions

Tl(I) and Tl(III) stock solutions were prepared as previously described (Hanzel and Verstraeten 2006). The amounts of Tl(I) or Tl(III) used in the experiments did not affect the pH of the culture medium.

Cell culture

Rat adrenal pheochromocytoma cells (PC12 cells) were chosen since they share similarities with sympathetic neurons, as they synthesize, store, and release catecholamines (Chen et al. 1994). Also, PC12 cells are widely accepted as a model of nerve cells, and they are extensively used for neurotoxicology and neuronal differentiation studies (Fujita et al. 1989; Greene and Tischler 1976). PC12 cells were obtained from the American Type Culture Collection (A.T.C.C., Rockville, MD, USA) and cultured as previously described (Hanzel and Verstraeten 2006; Hanzel and Verstraeten 2009). As indicated for the individual experiments, cells were seeded on poly-L-lysinated 18-mm circle glass cover slips (5×10^5 cells), 60 mm (3×10^6 cells) Petri dishes, and 6- or 96-well plates

(1×10^6 and 3×10^4 cells/well, respectively) and allowed to grow until ~90 % of confluence. Culture media were replaced, and cells were added with Tl(I) or Tl(III) (10–100 μM) and further incubated as indicated for the individual experiments.

Endosomal acidification

The possible changes in the internal pH of the acidic compartments upon Tl exposure were evaluated by measuring the incorporation of the probe monodansylcadaverine (MDC) (Biederbick et al. 1995; Munafo and Colombo 2001). PC12 cells were grown on poly-L-lysinated 18-mm glass cover slips and incubated for 3 h in the presence of 100 μM Tl(I) or Tl(III). After incubation, culture media were removed, and cells were further incubated for 5 min at 37 °C in the presence of 50 μM MDC in phosphate buffer saline (PBS). Cover slips were washed 3 times with warm PBS, and samples were immediately observed through an Olympus BX50 fluorescence microscope (λ excitation, 360–370 nm; λ emission, 420–460 nm) coupled with a digital camera (Olympus Optical CO, LTD, Japan). MDC integrated optical density (IOD) per cell was measured using the routines available in Image-Pro Plus 5.1 (Media Cybernetics Inc., Bethesda, MD, USA).

Acridine orange uptake

Changes in the capacity of acidic compartments to maintain their internal pH upon Tl exposure were assessed by the incorporation of the fluorescent probe acridine orange (Yu et al. 2009). PC12 cells were grown on poly-L-lysinated 18-mm glass cover slips and incubated at 37 °C for 3 or 18 h in the presence of 10–100 μM Tl(I) or Tl(III). After incubation, culture media were removed, and cells were further incubated for 15 min in the presence of 5 μg/ml acridine orange in PBS. Cover slips were washed 3 times with warm PBS, and samples were immediately observed in a fluorescence microscope (λ excitation, 470–490 nm; λ emission, 510–550 nm). The ratio between red (late endosomes/lysosomes) and green (cytoplasmic and DNA-bound) fluorescence was calculated using the routines available in Image-Pro Plus 5.1.

Cathepsin D activity

Cathepsin D activity was evaluated as previously described (Yamamoto et al. 1979). PC12 cells were grown on 60-mm-diameter dishes and incubated at 37 °C for 6–24 h either in the absence or the presence of 100 μM Tl(I) or Tl(III). After incubation, cell lysates were obtained by incubating samples for 30 min at 4 °C in the presence of 0.05 M Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl, and 1 % (v/v) Triton X-100. The adequate amount of samples to obtain 10 μg of protein (Bradford 1976) was incubated at 30 °C for 4 h in the presence of 2.5 % (w/v) bovine hemoglobin in 0.2 M glycine (pH 3.1). Reaction was stopped by the addition of 1 ml of cold 20 % (v/v) TCA. After 10 min of incubation at 4 °C, samples were centrifuged at 800 $\times g$ at 4 °C for 10 min and supernatants were carefully transferred to 1.5-ml conical tubes. The content of acid-soluble peptides in samples was determined (Lowry et al. 1951) using tyrosine as standard. Results are expressed as the ratio between tyrosine and total proteins contents.

Cathepsin B activity

Portions of cell lysates obtained as described above and containing 100 μg proteins were added with 2 μl of the specific substrate Z-RR-pNA (0.2 mM final concentration) and/or 2 μl of the inhibitor E64d (10 μM final concentration), and final volume was adjusted to 0.2 ml with sodium phosphate buffer (pH 6.5) containing 2.5 mM EDTA and 2 mM DTT. Samples were further incubated at 37 °C for 30 min, and the release of p-nitroanilide was recorded every 30 min at 405 nm ($\varepsilon = 10{,}500\ M^{-1}\ cm^{-1}$) in a Biotrack II Microplate Reader (Biochrom Ltd., Eugendorf, Austria). Results are expressed as nmol of p-nitroanilide min^{-1} mg $protein^{-1}$.

Inmunofluorescence detection of cathepsin D, cathepsin B, and Lamp-1

PC12 cells were grown on 18-mm-diameter glass cover slip and incubated at 37 °C for either 6 or 18 h in the presence of 100 μM Tl(I) or Tl(III). Cells were washed 3 times with warm PBS and fixed with 4 % (w/v) paraformaldehyde in PBS containing 0.12 M sucrose, followed by 15-min incubation in the presence of 0.1 % (w/v) glycine in PBS. After washing, plasma membrane was permeabilized by incubating samples for 15 min in the presence of 0.1 % (v/v) Triton X-100 in PBS. Samples were incubated for 2 h with a blocking solution containing 5 % (v/v) bovine serum in PBS, followed by an overnight incubation at 4 °C in the presence of one of the following primary antibody mixtures: anti-cathepsin D (1:50 dilution) and anti-Lamp-1 (1:50 dilution), or anti-cathepsin B (1:50 dilution) and anti-Lamp-1 (1:50 dilution). After washing 3 times with PBS, samples were incubated for 2 h in the presence of a solution containing 5 μM Hoechst 32225, FITC-conjugated anti-rabbit antibody (1:200 dilution) (cathepsin D or B detection), and Texas Red-conjugated anti-goat antibody (1:200 dilution) (Lamp-1 detection). Cover slips were washed 3 times with PBS and mounted using Prolong® Gold antifade reagent. Cells were observed under an Olympus Fluoview FV1000 confocal microscope

(Olympus Corporation, Tokio, Japan) with objective lens USLSAPO 60× NA 1:35, magnification 600X. Fluorophores were detected using the following laser settings: FITC: λ excitation: 473 nm, λ emission: 519 nm, barrier filter (BF) 485, BF range 60 nm; Texas Red: λ excitation: 559 nm, λ emission: 612 nm, BF: FA575-675; Hoechst: λ excitation: 405 nm, λ emission: 461 nm, BF 425 nm, BF range 35 nm. Cells were counted to survey at least 100 cells per condition. The degree of colocalization of cathepsin D or B with the lysome marker Lamp-1 was evaluated from the changes in the overlap coefficient according to Manders et al. (1993), and the colocalization coefficients m1 and m2 were calculated as described by Zinchuk et al. (2005) using the routines available in Image-Pro Plus 5.1.

NADPH oxidase activity

NADPH oxidase activity was evaluated as described by Castilho et al. (1999). Cells were grown on 96-well plates apt for fluorescence measurements and incubated at 37 °C for either 1 or 3 h in the presence of Tl(I) or Tl(III) (10–100 μM). After incubation, culture media were removed, and cells were disrupted by 30-min incubation at 37 °C in the presence of 0.1 ml of Igepal 0.1 % (v/v). After the addition of 50 μl of a solution containing 0.3 mM dihydroethidine (DHE) and 1.5 mM NADPH in PBS, samples were further incubated at 37 °C for 1 h and the fluorescence intensity was recorded at 634 nm (λ excitation, 465 nm) in a PerkinElmer LS55 spectrofluorometer (Waltham, MA, USA) equipped with microplate fluorescence reader. Results were normalized by DNA content in the samples measured by reaction with 25 μM Hoechst 32258, and the intensity of the DNA-Hoechst complex was recorded at 420 nm (λ excitation, 370 nm).

Caspase 3 activity

Cells were grown on 6-well plates and incubated at 37 °C for 8 h with or without the addition of 100 μM E64d and/or 100 μM pepstatin A. The excess of inhibitors that was not incorporated into cells was removed by replacing the culture medium with fresh one, and cells were exposed for 18 h to 100 μM Tl(I) or Tl(III). Cells were collected by scrapping in warm PBS and centrifuged at 1,000×g for 10 min at 4 °C. Caspase 3 activity in the samples was assessed using the chromogenic substrate Ac-DEVD-pNA as previously described (Hanzel and Verstraeten 2009). Results are expressed as pmol of p-nitroanilide min^{-1} μg $protein^{-1}$.

Western blot analysis

PC12 cells were grown on 60-mm Petri dishes and exposed to 10-100 μM Tl(I) or Tl(III) for 1–24 h, and full-length and truncated BID (t-BID) were detected in whole cell lysates (Hanzel and Verstraeten 2009). Proteins (100 μg per lane) were separated by reducing 10 % (w/v) polyacrylamide gel electrophoresis and transferred to PVDF membranes. Colored molecular weight standards (GE Healthcare, Piscataway, NJ, USA) were ran simultaneously. Membranes were blocked for 1 h in 5 % (w/v) nonfat milk and incubated overnight at 4 °C in the presence of the corresponding primary antibody (1:1,000 dilution), followed by incubation with the corresponding peroxidase-conjugated secondary antibody (1:10,000 dilution). Specific bands were revealed by reaction with chemiluminescent ECL Western blotting detection reagent and detected in a Storm Phosphoimager 840 (Molecular Dynamics, Sunnyvale, CA, USA). Western blot data were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics Inc., Bethesda, MD, USA).

Inmunofluorescence detection of BID

PC12 cells were grown on 18-mm-diameter glass cover slip and incubated at 37 °C for 8 h in the presence of 100 μM E64d or pepstatin A. The excess of inhibitors that was not incorporated into cells was removed by replacing the culture medium, and cells were exposed for 18 h to either Tl(I) or Tl(III) (100 μM). Samples were processed as described above using the primary antibody anti-full-length BID (1:50 dilution) followed by incubation in the presence of the FITC-conjugated secondary antibody (1:200 dilution). Cell nuclei were labeled with 5 μM Hoechst 32258. After washing and mounting, cells were observed by fluorescence confocal microscopy. Lasers were set for the detection of FITC and Hoechst as described above. FITC-integrated optical density in the individual cells was quantified using the routines available in Image-Pro Plus 5.1.

Statistics

One-way analysis of variance (ANOVA) followed by Fisher's PLSD (protected least square difference) test was performed using the routines available in StatView 5.0 (SAS Institute, Cary, NC, USA). Two-way ANOVA was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, CA, USA). A probability (P) value < 0.05 was considered statistically significant.

Results

Tl(III) but not Tl(I) causes alterations in the acidic cell compartments

The possibility that Tl may induce the acidification of intracellular acidic compartments was evaluated in PC12

cells exposed for 3 h to Tl(I) or Tl(III) (100 μM) and labeled with the fluorescent probe MDC. When experiments were performed in the presence of complete medium containing 10 % (v/v) horse serum and 5 % (v/v) fetal bovine serum, the incorporation of MDC into Tl(III)-treated cells was 38 % higher ($P < 0.005$) than in control cells (Fig. 1). Under these conditions, Tl(I) did not affect MDC uptake. When the incubation media were replaced by a serum-free DMEM, neither Tl(I) nor Tl(III) altered MDC incorporation (Fig. 1). After the supplementation of serum-free DMEM with 0.35 mg/ml transferrin, the incorporation of MDC into Tl(III)-treated cells was 47 % higher than in controls ($P < 0.005$, ANOVA) (Fig. 1). The amount of transferrin used for the experiments was chosen based on the concentration of this protein in the complete culture media containing horse and fetal bovine sera that have approximately 3.3 and 0.4 mg/ml transferrin, respectively.

To assess whether Tl exposure could affect the capacity of lysosomes to maintain their internal pH, cells were incubated in the presence of acridine orange. When concentrated in lysosomes, this probe emits red fluorescence, with cells showing a granular pattern of labeling (Fig. 2a). In addition, this probe can be found in the cytosol and in nuclei, although emitting green fluorescence (Fig. 2a) (Zdolsek et al. 1993). Tl(I) (100 μM) did not affect lysosome labeling either after 3 or 18 h of incubation, with a red-to-green fluorescence ratio similar to that found in controls (Fig. 2b). Similar to the observation with MDC, after 3 h of incubation in the presence of 100 μM Tl(III), a tendency ($P = 0.09$) toward acridine orange accumulation in lysosomes was found. After 18 h of exposure to Tl(III), cells' capacity to concentrate the probe in their lysosomes decreased to 87 % ($P < 0.001$) (Fig. 2).

The damage to acidic compartments is involved in Tl(III)-mediated cell apoptosis

To evaluate whether the alteration in the acidic compartments upon Tl(III) exposure could be associated with the mobilization of certain lysosomal hydrolases that trigger cell apoptosis, the subcellular localization of cathepsins D and B was evaluated by immunofluorescence (Fig. 3a). After 6-h exposure of cells to 100 μM Tl(I), the colocalization of cathepsin D with the lysosome marker Lamp-1 decreased 15 % with respect to controls, while cathepsin B decreased only 8 % (Table 1). Tl(III) (100 μM) caused marked delocalization of both cathepsins, with a magnitude higher than that due to Tl(I) (Fig. 3a). Tl(III) caused a 27 and 32 % decrease in cathepsin D and B colocalization with Lamp-1, respectively (Table 1). When evaluated after 18 h of exposure to either 100 μM Tl(I) or Tl(III), the overlapping coefficients for the cathepsins and Lamp-1 were similar to those found in control cells (Table 1).

The effects of Tl(I) and Tl(III) on the activity of cathepsin D and B were also assessed. Cells that were exposed between 6 and 24 h to 100 μM Tl(I) or Tl(III) displayed higher cathepsin D activities than control cells, an effect that increased along the incubation period (Fig. 3b). In Tl(III)-treated cells, this effect was significant after 12 h of incubation ($P < 0.01$), while the effect of Tl(I) only reached significance after 24 h of incubation ($P < 0.05$). The effects of Tl(I) and Tl(III) on cathepsin D activity also depended on their concentration in the culture media as evaluated after 24-h exposure of cells to the cations (Fig. 3c). The magnitude of the effect due to Tl(III) was significant ($P < 0.05$) even at the lowest concentration assessed and was higher than the observed for Tl(I) ($P < 0.05$, two-way ANOVA). The activity of cathepsin B in Tl(I) (100 μM)-treated cells was not affected until 24 h of incubation ($P < 0.05$) (Fig. 3d). Similar to the observation with cathepsin D, 100 μM Tl(III) caused an early raise in cathepsin B activity, being this effect significant ($P < 0.01$) after 6 h of incubation, and sustained for at least 24 h of exposure to the metal (Fig. 3d).

In order to investigate whether the increase in cathepsins activities found in Tl-treated cells could be caused by an enhanced generation of reactive oxygen species, the activity of NADPH oxidase was evaluated by means of the oxidation of the fluorescent probe DHE. After 1 h (Fig. 4a) or 3 h (Fig. 4b) of exposure to Tl(I) (10–100 μM), the oxidation of DHE was slightly lower (range, 5–9 %) than in controls ($P < 0.01$, ANOVA). On the other hand, 1 h of exposure to Tl(III) did not affect DHE oxidation (Fig. 4a), and a moderate increase in DHE oxidation was observed after 3 h of incubation, an effect that depended on Tl(III) concentration in the culture media (Fig. 4b). The maximal DHE oxidation was achieved at 100 μM concentration of Tl(III) and corresponded to a 7.3 % increase with respect to the values measured in control cells ($P < 0.001$, ANOVA).

The possible participation of cathepsins in Tl(III)-mediated activation of caspase-dependent apoptosis was also investigated. For that purpose, cells were pre-incubated for 8 h in the presence of pepstatin A (cathepsin D inhibitor), E64d (cathepsin B inhibitor), or a combination of both and were further exposed to Tl for 18 h. This time point was chosen based on our previous results showing that the maximal activation of the intrinsic pathway of apoptosis is achieved after 18-h exposure of cells to Tl (Hanzel and Verstraeten 2009). Cell treatment with the combination of both inhibitors had no effects on caspase 3 activity per se (Fig. 5). In accordance with our previous results (Hanzel and Verstraeten 2009), in 100 μM Tl(I)- and Tl(III)-exposed cells, the activity of caspase 3 was 5.5- and 5.1-times higher than in controls, respectively ($P < 0.001$, ANOVA). Pepstatin A and E64d slightly prevented Tl(I)-mediated caspase

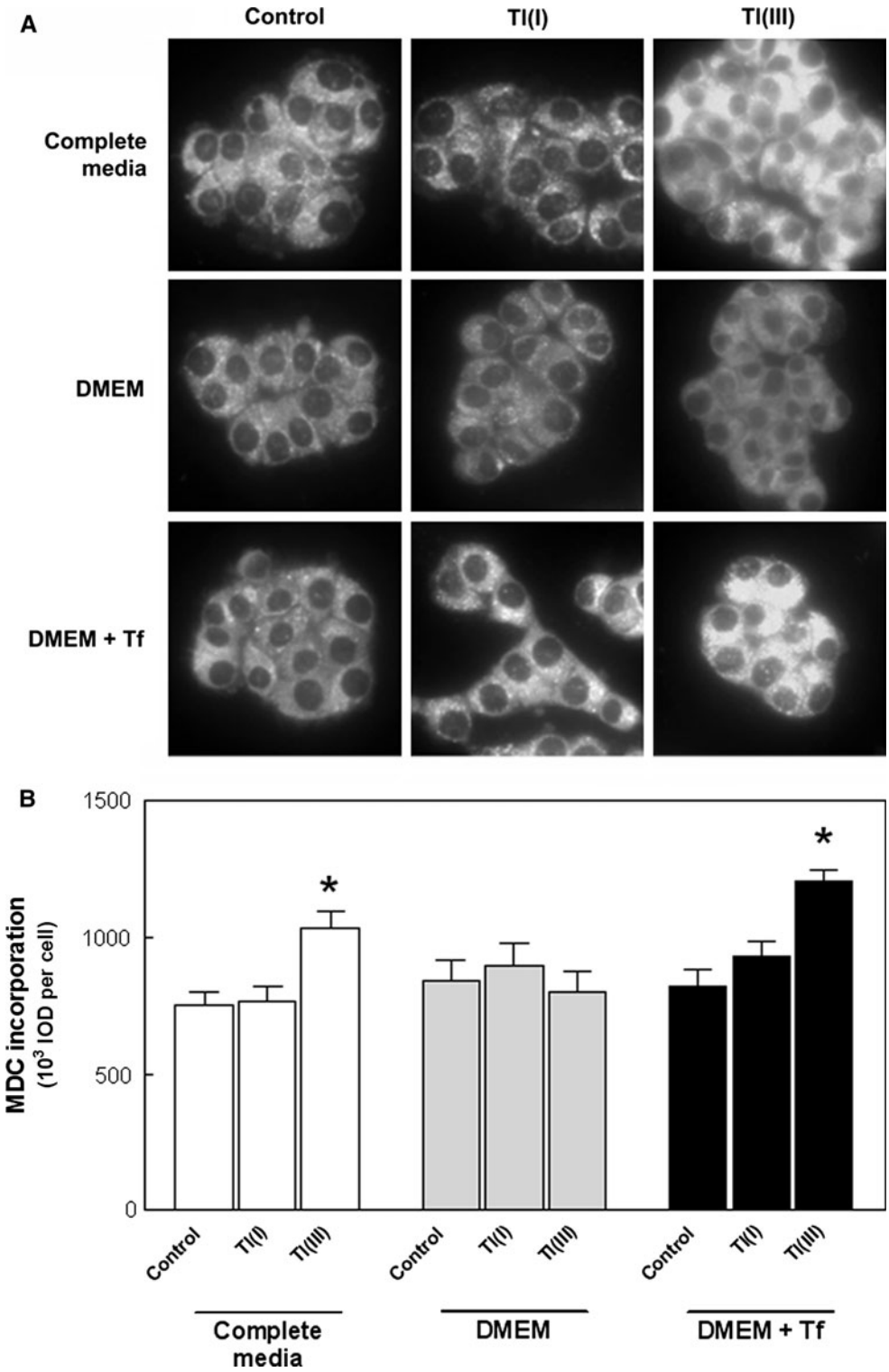

Fig. 1 Tl(III) but not Tl(I) causes the acidification of intracellular acidic compartments. PC12 cells were incubated at 37 °C for 3 h in the presence of 100 μM Tl(I) or Tl(III). After incubation, cells were labeled with the fluorescent probe MDC as described under "Materials and methods". **a** MDC incorporation observed by fluorescence microscopy in cells incubated for 3 h at 37 °C in a complete media (*top panel*), in serum-free DMEM (*central panel*), or in serum-free DMEM supplemented with 0.35 mg/ml transferrin (DMEM+Tf, *lower panel*), containing no further additions (*control*, *left panel*), 100 μM Tl(I) (*central panel*), or Tl(III) (*right panel*). Magnification: 600X. **b** Quantification of MDC incorporation in complete (*Open square*), DMEM (*dashed square*), and DMEM+Tf (*filled square*) media. Results are shown as the integrated optical density (IOD) per cell and are the mean ± SEM of four independent experiments. *Significantly different from the value measured in control cells for each experimental situation ($P < 0.005$, ANOVA)

3 activation (Fig. 5). On the contrary, in the presence of pepstatin A, the effect of Tl(III) was 60 % lower than the data obtained in the absence of the inhibitor ($P < 0.001$, ANOVA). The effect of E64d was only partial, preventing in a 25 % the activation of caspase 3 by Tl(III) ($P < 0.01$, ANOVA). The combination of both inhibitors had no additional effects on caspase 3 activation with respect to the data obtained by pepstatin A alone (Fig. 5). These results suggest that in Tl(III)-exposed cells, the release of cathepsin D, and to a minor extent cathepsin B, from lysosomes to cytosol participates in the activation of caspase-dependent apoptosis.

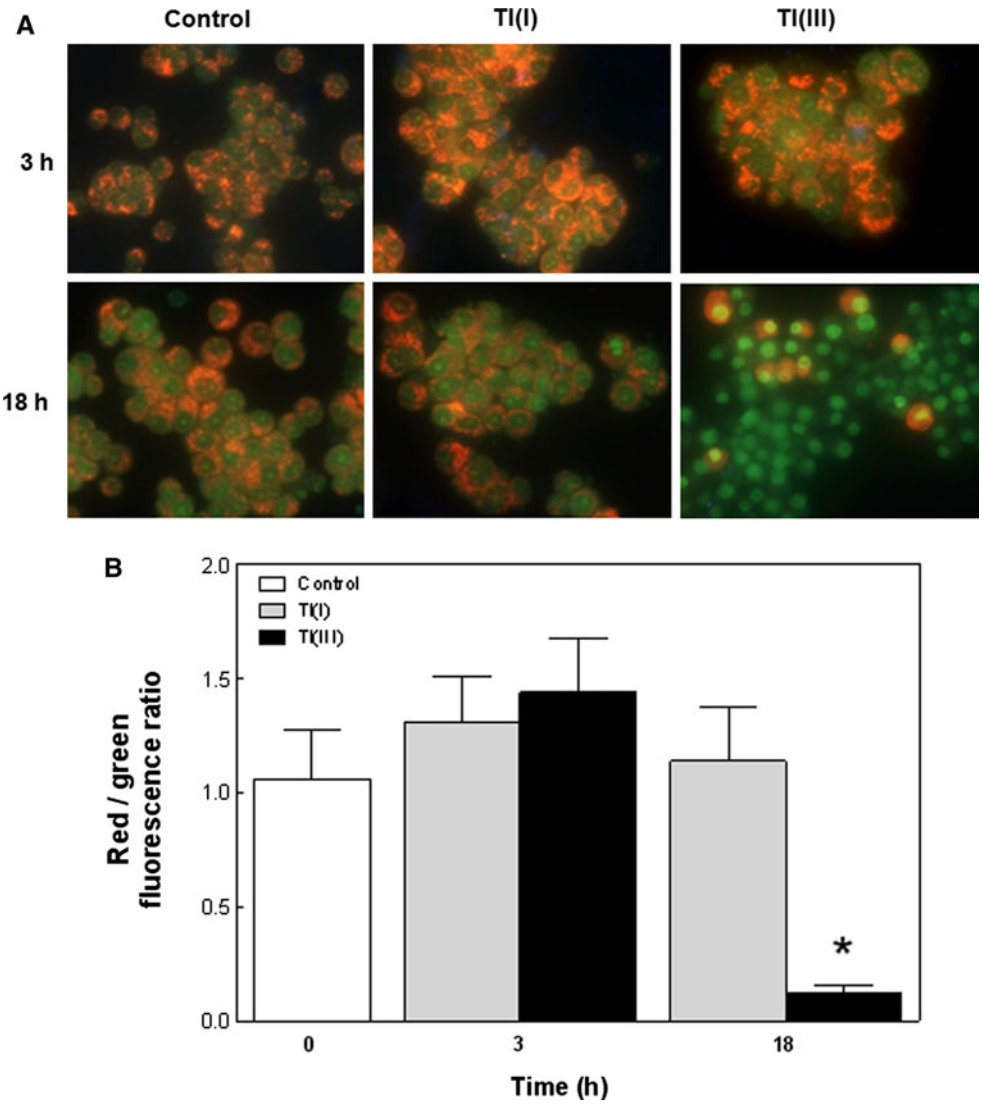

Fig. 2 Tl(III) but not Tl(I) affects the lysosomal uptake of acridine orange. PC12 cells were incubated at 37 °C for 3 or 18 h in the presence of 100 μM Tl(I) or Tl(III), and the uptake of the fluorescent probe acridine orange by lysosomes was investigated as described under "Materials and methods". **a** Representative fluorescence microscopy images showing the intracellular distribution of the probe (*granular red* fluorescence, lysosomes; diffuse green fluorescence, cytosol and DNA-bound). **b** Quantification of acridine orange fluorescence in control (*Open square*), Tl(I) (*dashed square*)-, and Tl(III) (*filled square*)-treated cells. Results are shown as the percentage of the value measured in controls and are the mean ± SEM of four independent experiments. *Significantly different from the value measured in control cells ($P < 0.001$, ANOVA) (color figure online)

Finally, the cleavage of the pro-apoptotic protein BID was evaluated in PC12 cells exposed for 1–24 h to 100 μM Tl(I) or Tl(III). BID cleavage is evidenced from the decrease in the band of 22 kDa corresponding to full-length BID with a concomitant appearance of a 15-kD band corresponding to the truncated form of BID (t-BID) (Fig. 6a). In Tl(I)-treated cells, a tendency ($P = 0.09$) toward high amounts of t-BID was observed, effect that reached statistical significance only after 24 h of exposure to the metal ($P < 0.05$) (Fig. 6b). On the other hand, cells incubated with 100 μM Tl(III) had higher contents of t-BID than controls, effect that was significant ($P < 0.05$) even at the shortest period of incubation assessed (Fig. 6b). The participation of cathepsins D and B on BID cleavage was evaluated by immunofluorescence using a primary antibody against full-length BID (Fig. 6c). After 18 h of exposure to 100 μM Tl(I), the immunostaining of BID was similar to that found in control cells (Fig. 6d). On the other hand, in Tl(III) (100 μM)-treated cells, the amount of BID was 41 % lower ($P < 0.05$) with respect to controls (Fig. 6d). The effect of Tl(III) on BID content was prevented in 30 % by pre-incubation of cells with E64d (Fig. 6d). On the other hand, pretreatment of cells with pepstatin A fully prevented the capacity of Tl(III) to promote the decrease in BID content (Fig. 6d).

Discussion

Participation of lysosomes in programmed cell death has gained importance in the last decade, as another mechanism able to initiate the cascade of events that leads to the activation of executioner caspases (Guicciardi et al. 2004). The list of agents that cause lysosomal destabilization is growing and includes both exogenous, such as photodamage and certain xenobiotics, and endogenous factors, such

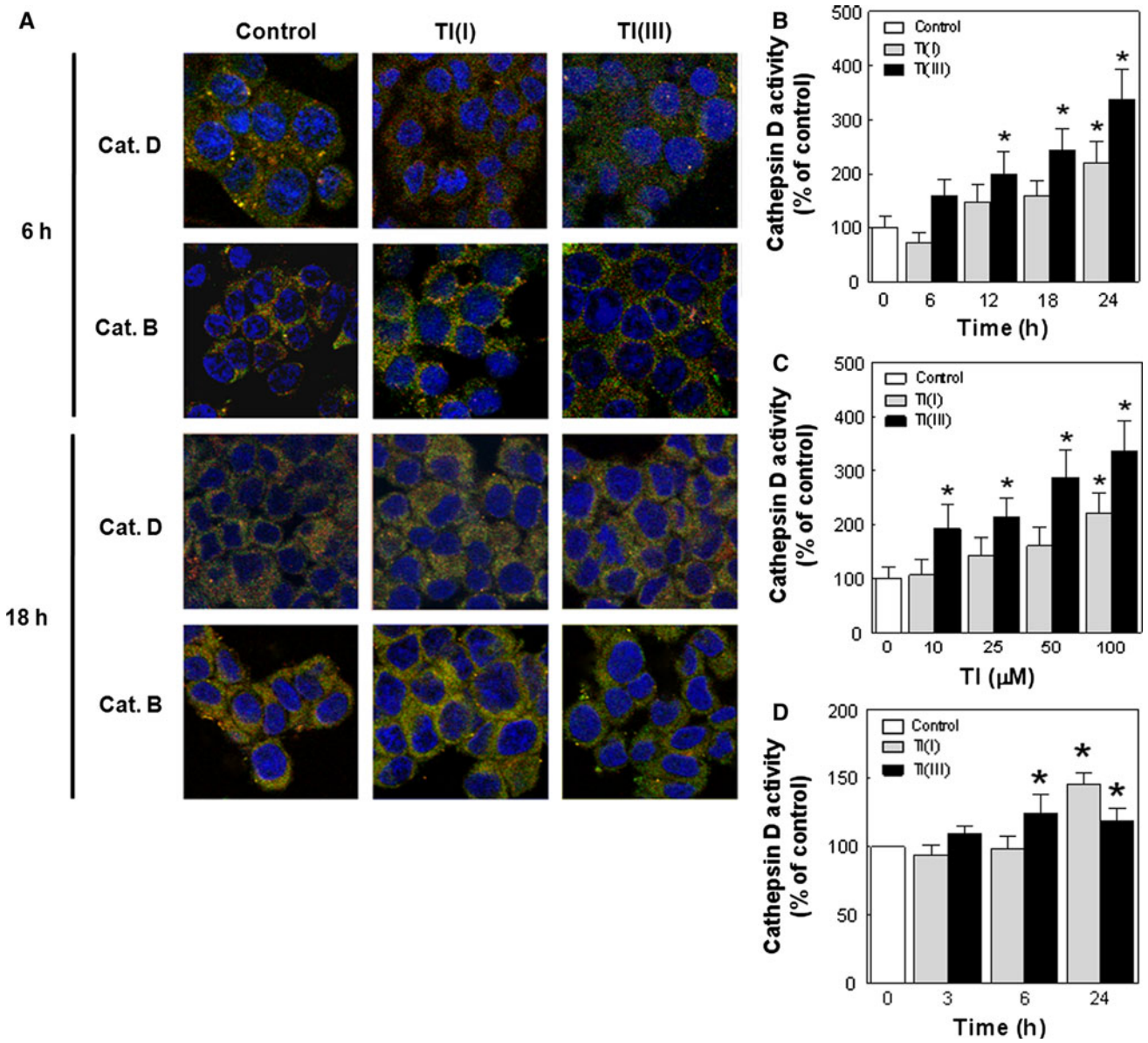

Fig. 3 Tl exposure affects the subcellular localization and activity of cathepsins D and B. **a** PC12 cells were incubated at 37 °C for 6 or 18 h in the presence of 100 μM Tl(I) or Tl(III) and cathepsin D, cathepsin B, and lysosome marker Lamp-1 were evidenced by immunofluorescence. *Green* fluorescence: cathepsin D or cathepsin B; *red* fluorescence: Lamp-1; *yellow* fluorescence: colocalization; *blue* fluorescence: cell nuclei stained with the DNA probe Hoechst 32258. Magnification: 600×. **b** Cathepsin D activity in PC12 cells incubated at 37 °C for 6–24 h in the presence of 100 μM Tl(I) (*dashed square*) or Tl(III) (*filled square*). **c** Cathepsin D activity measured in PC12 cells incubated at 37 °C for 24 h in the presence of 10–100 μM Tl(I) (*dashed square*) or Tl(III) (*filled square*). **d** Cathepsin B activity measured in PC12 cells incubated at 37 °C for 3–24 h in the presence of 100 μM Tl(I) (*dashed square*), or 100 μM Tl(III) (*filled square*). Results are shown as the percentage of the activity measured in control cells (*Open square*) and are the mean ± SEM of four independent experiments. *Significantly different from the value measured in controls ($P < 0.05$, ANOVA) (color figure online)

as sphingosine and reactive oxygen species, among others (Guicciardi et al. 2004). It is important to notice that massive destruction of lysosomes conducts to cell necrosis, while discrete damage to these organelles leads to apoptosis (Guicciardi et al. 2004). As a consequence of partial lysosomes disruption, proteolytic enzymes confined to these organelles reach cell cytosol and trigger apoptosis.

In a previous study, we have demonstrated that both the ionic species of Tl induce cell death by promoting the activation of the mitochondrial pathway of apoptosis in PC12 cells (Hanzel and Verstraeten 2009). In addition, Tl(III) stimulates the extrinsic pathway, as evidenced from the increase in Fas content and caspase 8 activation (Hanzel and Verstraeten 2009). Given the differences in the ionic properties of Tl(I) and Tl(III), it is reasonable to

Table 1 Colocalization parameters

Time	Cathepsin D/Lamp-1	Cathepsin B/Lamp-1
6 h		
Overlapping coefficient		
Control	0.79 ± 0.03	0.74 ± 0.01
Tl(I)	0.67 ± 0.02	0.68 ± 0.02
Tl(III)	0.56 ± 0.01	0.50 ± 0.03
m1		
Control	0.95 ± 0.02	0.97 ± 0.01
Tl(I)	0.75 ± 0.04	0.87 ± 0.04
Tl(III)	0.76 ± 0.02	0.78 ± 0.04
m2		
Control	0.91 ± 0.02	0.81 ± 0.04
Tl(I)	0.89 ± 0.02	0.69 ± 0.03
Tl(III)	0.80 ± 0.02	0.62 ± 0.05
18 h		
Overlapping coefficient		
Control	0.74 ± 0.02	0.75 ± 0.02
Tl(I)	0.74 ± 0.02	0.75 ± 0.02
Tl(III)	0.76 ± 0.01	0.71 ± 0.02
m1		
Control	0.93 ± 0.01	0.93 ± 0.02
Tl(I)	0.95 ± 0.01	0.94 ± 0.02
Tl(III)	0.95 ± 0.01	0.94 ± 0.02
m2		
Control	0.81 ± 0.03	0.81 ± 0.02
Tl(I)	0.85 ± 0.03	0.85 ± 0.02
Tl(III)	0.85 ± 0.02	0.85 ± 0.02

PC12 cells were exposed for either 6 or 18 h to 100 μM Tl(I) or Tl(III), and the colocalization of cathepsin D/Lamp-1 and cathepsin B/Lamp1 was evaluated by immunofluorescence as described under "Materials and methods". Overlapping coefficients were calculated according to Manders et al. (1993). m1: colocalization of the red (Lamp-1)-green (cathepsin) pair; m2: colocalization of the green (cathepsin)-red (Lamp-1) pair. Results are expressed as the mean ± SEM of 3 independent experiments

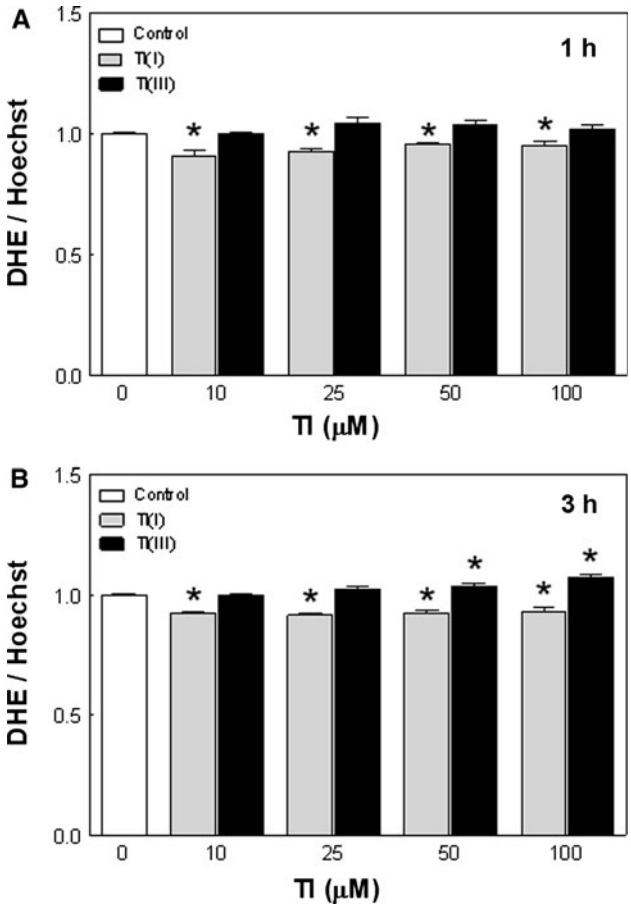

Fig. 4 Tl(I) and Tl(III) slightly affect NADPH oxidase activity. PC12 cells were incubated at 37 °C for **a** 1 h, or **b** 3 h in the absence (*Open square*) or presence of 10–100 μM Tl(I) (*dashed square*) or Tl(III) (*filled square*), and NADPH oxidase activity was evaluated from the oxidation of the probe DHE. Results are shown as the ratio between DHE and Hoechst 32258 fluorescence and are the mean ± SEM of five independent experiments. *Significantly different from the value measured in controls ($P < 0.01$, ANOVA)

hypothesize that both cations might be incorporated into cells following different routes. It has been described that the entry of Tl(I), a cation that resembles K^+ in its ionic properties, into glioma cells proceeds mainly through the Na^+-K^+-ATPase-dependent transport and/or the Na^+,K^+,Cl^- cotransport (Brismar et al. 1995; Sherstobitov et al. 2010). On the other hand, and based on its similarity with Fe^{3+}, Tl(III) may be able to bind to the main iron-transporting protein, transferrin. In fact, it has been demonstrated in vitro that Tl(III), but not Tl(I), strongly binds to transferrin with a binding constant of approximately 10^{22} (Li et al. 1996). Tl(III)–transferrin complex is very stable and prevents Tl(III) reduction to Tl(I) in the extracellular milieu (Bertini et al. 1983), thus increasing the availability of the trivalent cation to tissues and organs. If Tl(III) entry into cells proceeds through the internalization of a Tl(III)–transferrin complex, a decrease in endosomal pH is expected (Richardson et al. 2010). As suspected, Tl(I) did not modify the acidity of late endosome/lysosome compartments evidenced from a lack of change in MDC uptake. On the other hand, Tl(III) markedly acidified these compartments, an effect that is compatible with the internalization of a metal–transferrin complex. MDC is a widely used probe for the labeling of lysosomes, endosomes, amphisomes, and autophagosomes (Vazquez and Colombo 2009). Since the incubations were performed in the presence of 10 % horse serum and 5 % fetal bovine serum, cells were not in a starving state and therefore the labeling of autophagosomes and amphisomes may be discarded under the current experimental conditions.

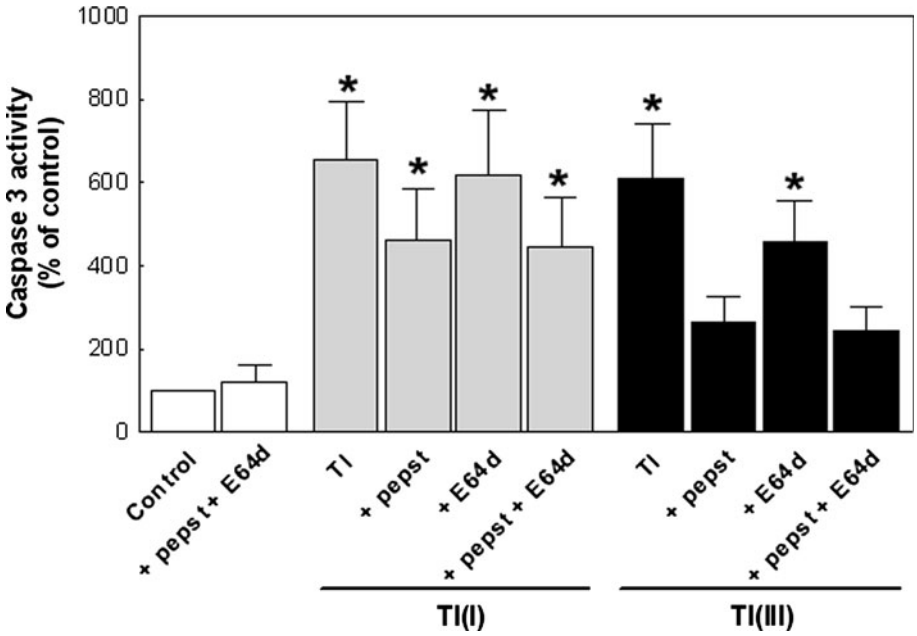

Fig. 5 Cathepsins inhibition reduces Tl(III)-mediated caspase 3 activation. PC12 cells were incubated at 37 °C for 8 h in the presence of 100 μM pepstatin A (pepst), 100 μM of E64d, or both (pepst + E64d). After the replacement of culture media, cells were further incubated at 37 °C for 18 h in the presence of 100 μM Tl(I) (*dashed square*) or Tl(III) (*filled square*), and caspase 3 activity was measured as described under "Materials and methods". Results are shown as the percentage of the activity in control cells (*Open square*) and are the mean ± SEM of four independent experiments. *Significantly different from the value measured in controls ($P < 0.01$, ANOVA)

To further explore the possibility that transferrin may be involved in Tl(III) uptake, experiments were also performed in the absence of serum to avoid transferrin in the medium. Under these conditions, no changes in the acidity of the acidic compartments were observed after 3 h of incubation in the presence of the cation. The supplementation of serum-free DMEM with apotransferrin resulted in increased intensity of MDC labeling, reaching similar levels to that obtained in cells incubated in the presence of serum-containing medium. Combined, these results support the hypothesis that in the current experimental conditions, MDC did not label autophagosomes or amphisomes, since no MDC uptake was evidenced in a protein-deprived medium, such as DMEM, but when either a complete media or transferrin-supplemented DMEM was used in the experiments.

The relevance of finding an acidification of late endosomes/lysosomes in Tl(III)-exposed cells is twofold. To our knowledge, this is the first report demonstrating that transferrin is actually involved in Tl(III) uptake in cultured cells, since until now all the studies were performed in cell-free systems (Harris and Messori 2002 and references therein). And secondly, based on the high redox potential of Tl(III), it is presumable that this cation may oxidize components of the extracellular milieu and the generated Tl(I) will be next taken up by cells. Therefore, the present results suggest that the trivalent cation can reach cell interior and trigger its toxic effects within cells. The fate of Tl(III) once it reaches cell interior remains to be elucidated.

Following the initial stage of acidification of the acidic compartments due to Tl(III)–transferrin uptake, lysosomes failed to keep their internal pH as evidenced from their poor ability to incorporate acridine orange. The maintenance of pH in lysosomes is mediated by a vacuolar-type H^+-ATPase (Smith et al. 2003), an enzyme that is a potential target of Tl(III) noxious actions. Alterations in the gradient of protons in lysosomes result in the disturbance of their membrane integrity and allow the exiting of hydrolytic enzymes (i.e., the cathepsins) toward the cytosol (Brunk et al. 1997). Even when these proteolytic enzymes require an acidic pH for optimal activity, they are still active at neutral pH and able to hydrolyze proteins in the cytosol (Guicciardi et al. 2004; Kagedal et al. 2001). In the present model, we found that Tl(III) promotes an early release of both cathepsin D and B from the lysosomes, as evidenced from the lower colocalization degree with lysosome marker Lamp-1 found in Tl(III)-treated cells after only 6 h of incubation. However, when cells were exposed to Tl(III) for a longer period (18 h), the colocalization between the cathepsins and Lamp-1 was similar to that found in control cells.

Interestingly, in Tl-treated cells, the total activities of cathepsin B and cathepsin D were increased with respect to controls. Working with cultured human foreskin fibroblast, Kagedal et al. (2001) observed that naphthazarine caused an oxidative stress status that resulted in cathepsin release to cytosol followed by an increase in their total activity. In that cell model, the oxidative stress enhanced the expression of p53 which is a transcription factor for cathepsin D (Kagedal et al. 2001). A similar response was observed in PC12 cells exogenously exposed to micromolar concentrations of H_2O_2 (Lee et al. 2007). We propose that this mechanism may be operative in our experimental model since both Tl(I) and Tl(III) cause an early increase in H_2O_2 concentration (Hanzel and Verstraeten 2006). In addition, the magnitude of the effect on H_2O_2 production is higher

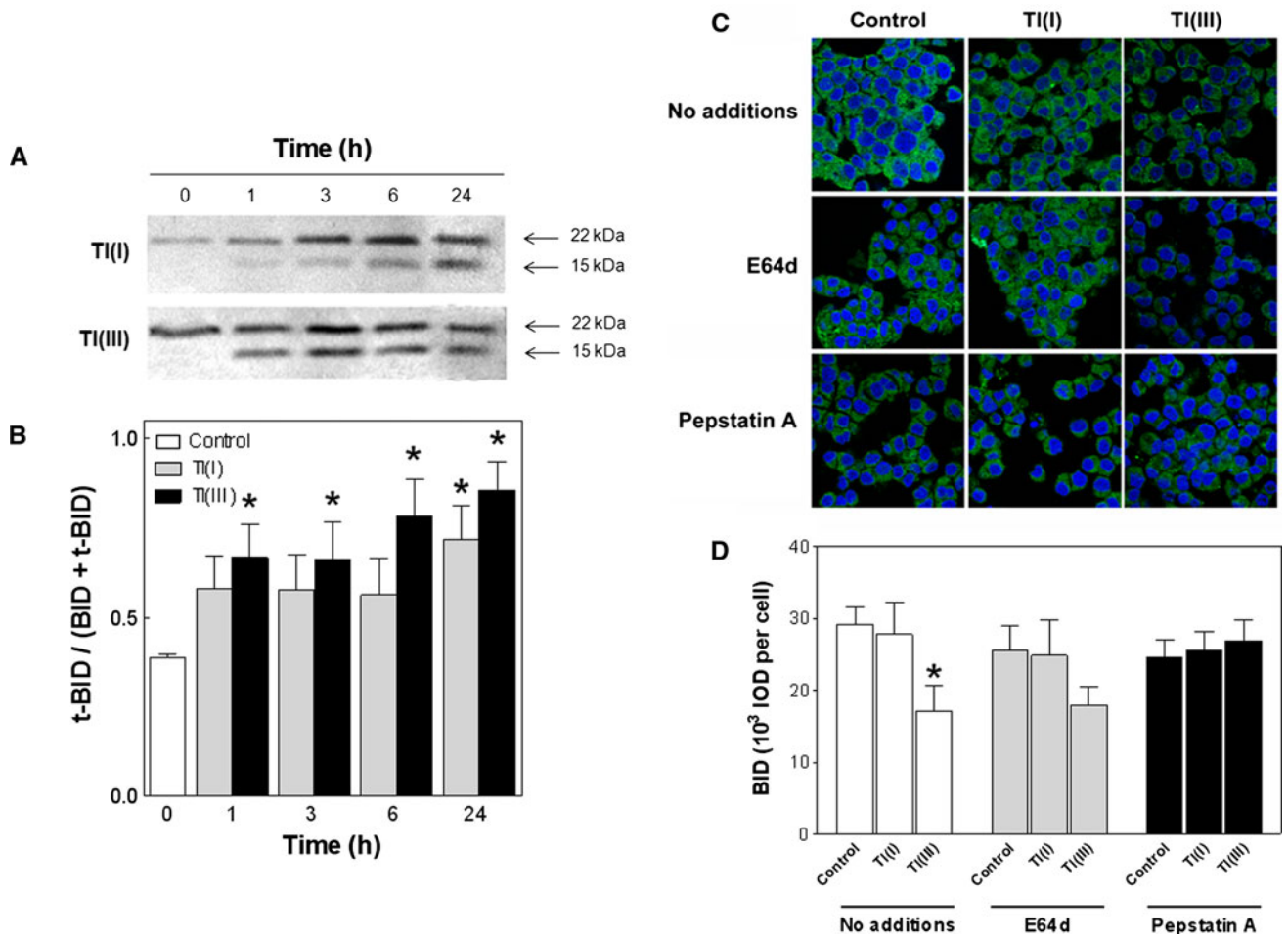

Fig. 6 Tl induces BID cleavage. PC12 cells were incubated at 37 °C for 1–24 h either in the absence or in the presence of 100 μM Tl(I) or Tl(III). After incubation, full-length (22 kDa) and truncated BID (t-BID, 15 kDa) were evidenced in the cell lysates. **a** Representative Western blot; **b** quantification of the cleaved (tBID) to total (BID+tBID) BID in control (*Open square*), Tl(I) (*dashed square*)- and Tl(III) (*filled square*)-treated cells. Results are shown as the t-BID-to-total BID ratio and are the mean ± SEM of five independent experiments. *Significantly different from the values measured in controls ($P < 0.01$, ANOVA). **c** PC12 cells were incubated at 37 °C for 8 h in the presence of E64d or pepstatin A and further incubated for 18 h in the presence of Tl(I) or Tl(III), and the expression of full-length BID was evaluated by immunofluorescence. *Green* fluorescence: BID; *blue* fluorescence: cell nuclei stained with the DNA probe Hoechst 32258. Magnification: ×600. **d** Quantitation of BID fluorescence in cells incubated in the absence of inhibitors (*Open square*), or in the presence of E64d (*dashed square*) or pepstatin A (*filled square*). Results are shown as the integrated optical density (IOD) per cell and are the mean ± SEM of five independent experiments. *Significantly different from the value measured in control cells ($P < 0.001$, ANOVA) (color figure online)

for Tl(III) than for Tl(I), which might be related to the differential effect found for Tl(III) in cathepsin release and activation. H_2O_2 is generated in mitochondria but also in the cytosol from the dismutation of superoxide anion by the enzyme superoxide dismutase. The main source of superoxide anion in cytosol is the enzyme NADPH oxidase, an enzyme that is activated as a response to cell contact with certain metals, such as arsenite, zinc, and transition metal–containing particles (Smith et al. 2001; Becker et al. 2002; Matsunaga et al. 2005). In spite of NADPH oxidase being active in Tl(III)-treated cells, the low magnitude of this effect (~7 % increase) makes this finding barely relevant from a biological point of view and allows us to discard the participation of this enzyme in lysosome destabilization. Finally, H_2O_2 can be generated from the redox-active iron stored in lysosomes that is released to cytosol under lysosome destabilizing conditions (Kurz et al. 2004; Tenopoulou et al. 2005).

The cysteine protease cathepsin B is known to cleave the pro-apoptotic protein BID, generating a fragment (t-BID) that translocates to mitochondria, promotes cytochrome c release from this organelle, and triggers the activation of the executioner caspases (Cirman et al. 2004). Cathepsin B-dependent BID cleavage is not the only

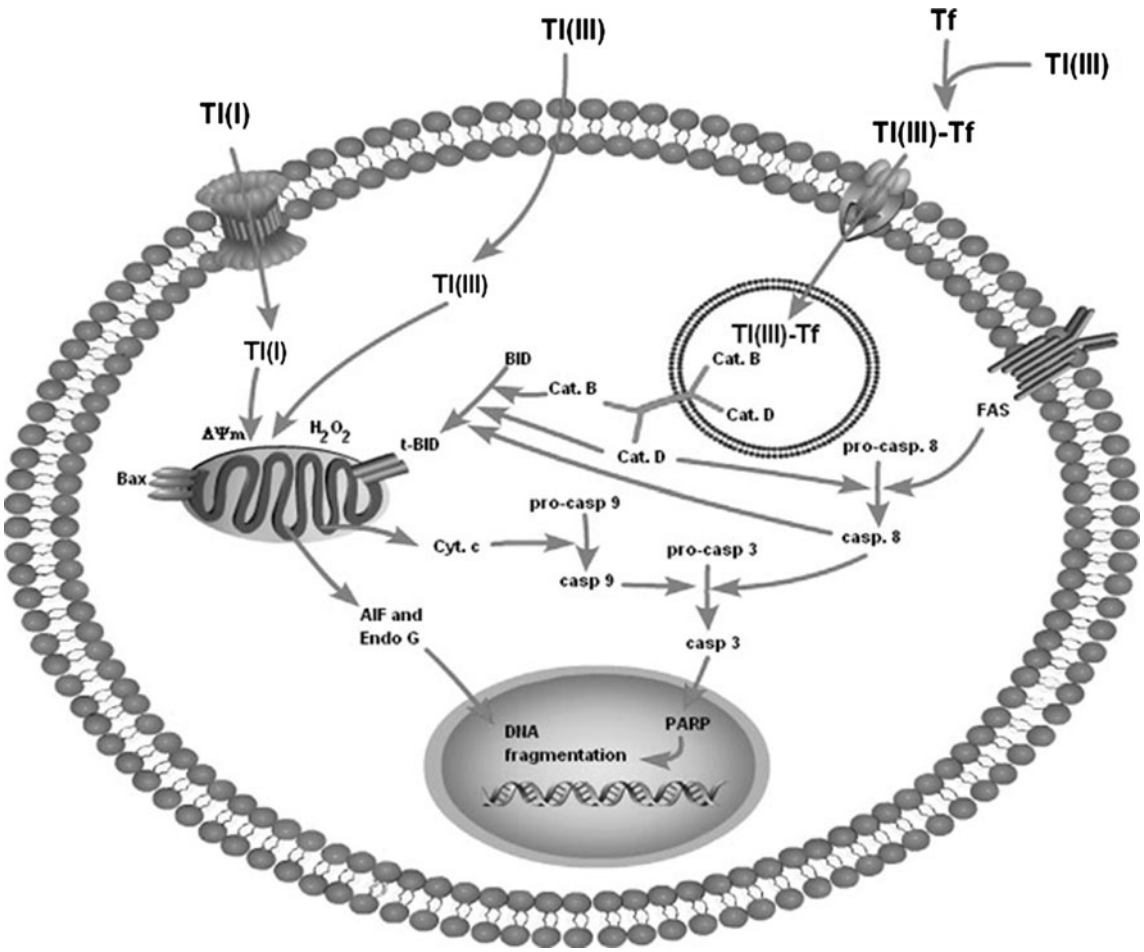

Fig. 7 Proposed sequence of events leading to Tl(I)- and Tl(III)-mediated apoptosis in PC12 cells. For details see text. *Dashed arrows* indicate still nonverified steps. ΔΨm: mitochondrial transmembrane potential; *cat. B* cathepsin B, *cat. D* cathepsin D, *cyt. c* cytochrome c, *Tf* transferrin, *AIF* apoptosis-inducing factorm, *Endo G* endonuclease G, *pro-casp* pro-caspase, *casp* caspase

mechanism that triggers apoptosis by generating t-BID. The aspartate protease cathepsin D also acts on this protein through two different mechanisms. When in the cytosol, the mature 34-kDa form of cathepsin D is able to hydrolyze BID (Liaudet-Coopman et al. 2006; Jaattela et al. 2004; Kagedal et al. 2001). Also, cathepsin D cleaves pro-caspase 8 rendering the proteolytically active fragment, caspase 8 (Conus et al. 2008). The active caspase 8 acts on BID generating t-BID (Li et al. 1998). The activation of cathepsin D seems to play a key role in Tl(III)-mediated apoptosis. Supporting this, its inhibition by pre-treating cells with pepstatin A prevented in a 60 % the activation of the executioner caspase 3 due to Tl(III), with a negligible effect on Tl(I)-exposed cells. On the other hand, E64d only caused a partial (25 %) inhibition of caspase 3 activation in cells exposed to Tl(III) without modifying the effect observed in Tl(I)-treated cells. This compound is a cysteinyl protease inhibitor and acts on cathepsin B, H, and L, and the nonlysosomal protease calpain (Tsubokawa et al. 2006). Therefore, the measured effect of E64d on Tl(III)-mediated caspase 3 activation resulted from the inhibition of all these pro-apoptotic enzymes as well. In spite of this, the magnitude of the inhibition is still lower than the observed for cathepsin D inhibition, stressing the relevance of this enzyme in Tl(III)-mediated apoptosis.

On the basis of our previous (Hanzel and Verstraeten 2006; Hanzel and Verstraeten 2009) and current results, we propose the following sequence of events to explain Tl-mediated PC12 cell apoptosis (Fig. 7). Tl(I) reaches cells interior following K^+ entrance and affects mitochondria. By decreasing mitochondrial membrane potential, Tl(I) promotes H_2O_2 production and the release of cytochrome c. Together with pro-caspase 9, Apaf-1 (apoptosis protease activating factor 1), and dATP, cytochrome c forms a multiprotein complex called apoptosome that activates caspase 9, leading to activation of the final steps of apoptosis. In addition, other caspase-independent apoptotic proteins are released from mitochondria, such as the apoptosis-inducing factor (AIF) and endonuclease G (EndoG). Moreover, long-term H_2O_2 generation by

Tl(I) will damage lysosomes. On the other hand, Tl(III) reaches cells following Fe-transferrin route, and the cation accumulates in the acidic compartments. By destabilizing their membranes, Tl(III) facilitates the release of pro-apoptotic hydrolytic enzymes, such as the cathepsins. Particularly, cathepsin D cleaves BID and the product t-BID homoolygomerizes in the mitochondrial membrane, allowing the release of cytochrome c (Grinberg et al. 2002). Also, t-BID helps the oligomerization of Bax (Bcl-2-associated X protein) in the mitochondrial membrane and facilitates the exit of mitochondrial proteins into cytosol (Terman et al. 2006). This is an operative mechanism in our experimental model, since Tl(III), and to a lesser extent Tl(I), enhances Bax oligomerization (Hanzel and Verstraeten 2009).

In addition, and through a still not elucidated mechanism, Tl(III) induces the extrinsic pathway of apoptosis, with the consequent activation of caspase 8 that may act simultaneously on pro-caspase 3 and BID. The possibility that Tl(III) may also enter cells through a different mechanism cannot be ruled out at this point and deserves further investigation. It is also possible that upon lysosomal destabilization, Tl(III) may be released to the cytosol either as the trivalent cation or as its reduced form, Tl(I). In this case, the cation will be free to interact with other organelles, such as mitochondria, and triggers the intrinsic pathway of apoptosis. In summary, experimental evidence suggests that not only mitochondria but also lysosomes are major targets for Tl(III) noxious effects and partially responsible for deleterious effects of this cation on cell viability.

Acknowledgments This work was supported by grants of the University of Buenos Aires (B086), CONICET (PIP 112-200801-01977), and ANPCyT (PICT 32273), Argentina. SVV is a career investigator of the CONICET (National Research Council, Argentina).

Conflict of interest The authors declare that there are no conflicts of interest.

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