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Cytotoxic effects of crotamine are mediated through lysosomal membrane permeabilization

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ABSTRACT

Crotamine, one of the main toxic components of *Crotalus durissus terrificus* venom, is a small non-enzymatic basic polypeptide, which causes hind limb paralysis and necrosis of muscle cells. It is well-known that several toxins penetrate into the cytosol through endocytosis, although in many cases the mechanism by which this occurs has not been fully investigated. Recently, using low concentrations of crotamine, we demonstrated the uptake of this toxin into actively proliferative cells via endocytosis, an event that ensues crotamine binding to cell membrane heparan sulfate proteoglycans. Thus, crotamine can be regarded as a cell-penetrating peptide that, additionally, has been shown to be able of delivering some biologically active molecules into various cells. Herein, we investigate one of the mechanisms by which crotamine exerts its cytotoxic effects by following its uptake into highly proliferative cells, as CHO-K1 cells. Crotamine accumulation in the acidic endosomal/lysosomal vesicles was observed within 5 min after treatment of these cells with a cytotoxic concentration of this toxin, a value determined here by classical MTT assay. This accumulation caused disruption of lysosomal vesicles accompanied by the leakage of these vesicles contents into the cytosol. This lysosomal lysis also promoted the release of cysteine cathepsin and an increase of caspase activity in the cytoplasm. This chain of events seems to trigger a cell death process. Overall, our data suggest that lysosomes are the primary targets for crotamine cytotoxicity, a proposal corroborated by the correlation between both the kinetics and concentration-dependence of crotamine

Abbreviations: CPP, Cell penetrating peptide; AO, Acridine orange; CHO, Chinese hamster ovary; Cy3-crotamine, Cy3-conjugated crotamine; FITC-crotamine, FITC-conjugated crotamine; Fcm, Fluorescent Confocal Microscopy; DIC, Differential Interference Contrast; FBS, Fetal bovine serum.

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accumulation in lysosome compartments and the cytotoxic effects of this protein in CHO-K1 cells. Although crotamine is usually regarded as a myotoxin, we observed that intraperitoneal injection of fluorescently labeled crotamine in living mice led to significant and rapid accumulation of this toxin in the cell cytoplasm of several tissues, suggesting that crotamine cytotoxicity might not be restricted to muscle cells.

1. Introduction

Little is known about toxins distribution within the tissues at the cellular level, about their penetration through cellular membranes, and even less about their intracellular and subcellular localization following accidental or experimental snake envenomation. Data about the ability of any particular toxin to penetrate tissues and cells and to interact with subcellular structures will certainly provide important clues regarding the mechanisms involved in the toxic effects of these compounds. Eventually, they will also open new perspectives for the use of such venom components in biomedicine, as it has been described for a number of other natural toxins (Ménez, 1998; Pal et al., 2002). These studies will not only complement our understanding on the mechanisms of the toxic effects of the venom components at the entire organism level, as well as at tissue and cellular levels, but it may also contribute to complement our current knowledge about the physiological functioning of the cells (Brazil and Fontana, 1993; Ownby, 1998; Ohkura et al., 1994).

Crotamine is a highly basic toxin originally described as the main component of the South American rattlesnake venom responsible for the hind limb paralysis observed in mice after venom injection (Gonçalves and Vieira, 1950). Its primary structure is nearly identical to those of other small, non-enzymatic, myonecrotic toxins found in different rattlesnake venoms. The myotoxicity mechanisms of crotamine and the closely related myotoxin-*a* have been both unraveled in part by electrophysiological and biochemical studies carried out in different isolated muscle preparations or cultured muscle cells. It has been shown that these toxins cause reduction in the resting membrane potential and increase in membrane conductance by a tetrodotoxin-sensitive, i.e., Na^+-channel mediated mechanism (Hong and Chang, 1985; Brazil and Fontana, 1993). However, recently described electrophysiological experiments showed that crotamine is inactive on sodium channels, suggesting that the actual mechanism of action of this toxin is still a matter of debate (Rizzi et al., 2007).

Crotamine size, primary sequence, and positive charge, led us to investigate its cell-penetrating activity using this toxin at concentrations shown to be non-toxic to the studied cells (Kerkis et al., 2004). We showed that crotamine belongs to a group of natural cell-penetrating peptides (CPPs), which are highly cationic with low molecular weight, rich in basic amino acids residues, such as, arginine or lysine, or proline-rich peptides (Kerkis et al., 2006). Furthermore, we demonstrated that crotamine, in a condition presenting no signals of toxicity, can be used as a carrier of negatively charged molecules into the cells through binding to cell membrane heparan sulfate proteoglycans followed by endocytosis (Nascimento et al., 2007). Herein, we have determined the cytotoxic concentration of crotamine in CHO-K1 cells by classical MTT assay and, using the determined IC_{50} concentration of crotamine, we evaluated the effect of this toxin after penetration into these actively proliferative cells by monitoring the lysosomal targeting and leakage, followed by the release of cysteine cathepsins and caspase activation, which can trigger the cell death process. Although muscles are considered the main target of crotamine toxic effect after envenomation, intraperitoneal injection of labeled toxin in living mice, allowed to observe cytoplasmic localization of this toxin in several mouse tissues, suggesting that other cell types in addition to the skeletal muscle cells might be also involved in the toxicity of crotamine.

2. Materials and methods

2.1. Materials

Cell culture medium and supplements were from Invitrogen (Gaithersburg, MD, USA). *Crotalus durissus terrificus* venom was extracted from snakes maintained at the Faculdade de Medicina de Ribeirão Preto (FMRP) serpentarium, São Paulo University, and crotamine was obtained essentially as previously described (Kerkis et al., 2004). All chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell viability assay

Cell viability following crotamine exposure was examined using an MTT assay. Metabolically active mitochondrial dehydrogenases convert the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Chemical Co., St. Louis, MO, USA) to insoluble purple formazan crystals at a rate that is proportional to cell viability. The cultured CHO-K1 cells (100 μl) were plated in 96-well microtitre plates at a concentration of 2×10^5 cells/ml. After overnight incubation, the medium was removed and the cells were incubated with appropriate amounts of the toxin in the presence of 100 μl culture media supplemented with 1 or 10% of fetal bovine serum (FBS) for 24 h, at 37 °C, in an atmosphere of 5% CO_2 in air. For the treatment with the E-64 inhibitor, 5 μM of the inhibitor with 0.001% of saponin was added 12 h before starting the treatment for 24 h with several concentrations of crotamine (0–40 μM). At the end of incubation, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well. After 4 h, acid-isopropanol (100 μl of 0.04 N HCl in isopropanol/well) was added and mixed thoroughly, and the plate was read on a FlexStation 3™ (Molecular Devices, Sunnyvale, CA, USA, www.moleculardevices.com), using a test wavelength of 570 nm and a reference wavelength of

620 nm (MTT viability assay). Percent of cytotoxicity was calculated as $100 \times (1 - [\text{optical density at 570–620 nm with the toxin}]/[\text{optical density at 570–620 nm without the toxin}])$.

2.3. Labeling of crotamine with fluorescent dye

Fluorescent crotamine derivatives were prepared by using the Fluorolink™ Cy3-reactive dye (GE Healthcare UK Limited, Amersham Place, England) or the FluoReporter® FITC Protein Labeling Kit (Molecular Probes, Netherlands), following the instructions of the respective manufacturers. After labeling, the remaining free fluorescent dye was eliminated by using a filtration device with a cut-off of 3000 Da (Centricon 3 MWCO concentrator, Amicon, Millipore Co., USA). The degree of labeling was estimated by absorbance measurements as indicated by the dye manufacturers and confirmed by MALDI-TOF mass spectrometry analyses (TofSpec-E, Micromass, UK).

2.4. Lysossomal localization of FITC-crotamine in CHO-K1 cells

The endocytic acidic compartments of the CHO-K1 living cells were labeled with 1 nM Lyso Tracker DND-99, for 30 min, at 37 °C. Thereafter, the cells were washed with F-12 medium and incubated with FITC-crotamine (0.5–5 μM). The fluorescent signal of Lyso Tracker DND-99 and FITC-crotamine as well as the phase contrast micrographs were monitored at real time with confocal laser scanning microscope.

2.5. Lysosomal leakage assays

Lysosomal/endosomal leakage of CHO-K1 cells induced by crotamine internalization was monitored using vital acridine orange (AO) as described previously (Robbins and Marcus, 1963). For imaging, CHO-K1 cells were grown on cover glasses and the living cells were labeled with 5 μg/ml of AO in F-12 medium, for 15 min, at 37 °C. Thereafter, the cells were washed with F-12 medium, and then exposed to 5 μM crotamine for different periods at 37 °C. The fluorescent signals of AO were taken with a confocal microscope.

2.6. Confocal microscopy assays

LSM 501 (Carl Zeiss, Jena, Germany) confocal microscopy was used to acquire the images that were analyzed by LSM 5 Image Examiner software. The following configurations were used: objective Plan-Neofluar 40x/1.3 Oil; Pinhole: 2.55 Airy Units; Frame Size: 1024 × 1024; Scan Speed: 11 (Pixel Time: 0.96 μs and Scan Time: 1.77 s). FITC-crotamine was excited with Argon Laser at 488 nm and the green light was captured by band pass (BP) 500–550 nm filter. Lyso Tracker DND-99 labeling endosomes/lysosomes were excited by HeNe1 laser at 543 nm and the red light was captured with long pass (LP) 560 nm filter. Differential Interference Contrast (DIC) images were performed using HeNe2 laser at 633 nm. AO assay was performed using Argon laser at 488 nm excitation and the emission light varied between green (BP 500–550 nm filter), for low dye concentration, and red (LP 560 nm filter), for higher concentrations of dye.

2.7. Lysosomal cysteine cathepsins released assays

For assay of lysosomal cysteine cathepsins activity released by the crotamine treatment, triplicate samples of supernatant cellular medium (100 μl) obtained after treatment with 0.001% of saponin were dispensed into a 96-well, black flat-bottomed, microfluor plate (Thermo Labsystems, Milford, MA, USA). The assay was initiated by addition of 100 μl of a solution containing 100 mM sodium acetate pH 5.5, 200 mM NaCl, 4 mM EDTA, 10 mM dithiothreitol, and 10 μM Z-FR-AMC. The production of AMC was continuously monitored for 20 min, at 37 °C, on a SpectraMAX Gemini microplate reader (Molecular Devices, Sunnyvale, CA, USA) employing excitation/emission wavelengths of 380/460 nm, respectively. Analyses performed in the presence of 5 μM E-64 inhibitor were used to estimate contributions of cysteine cathepsins activity to Z-FR-AMC cleavage. Activities measured in the supernatants of cultures incubated only with the release medium were used to correct for spontaneous release of enzymes (Caruso et al., 2006).

2.8. Evaluation of caspase activity

In order to determine the effect of crotamine uptake upon caspases activation about 10^6 CHO-K1 cells was treated or not with 5 μM crotamine by 24 h in F-12 medium. The activity of caspases was determined using the caspase 3, 6 or 8 fluorometric assay kits (Sigma, Saint Louis, Missouri, USA), following the manufacturer's instructions. Briefly, the activity was determined by the release of fluorescent coumarinic compounds in a HITACHI F-2500 fluorescence spectrophotometer equipped with a thermostatic cell holder using $\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm. The activities were monitored continuously during 90 min. Cell lysates were incubated at 37 °C with the selective substrates: Ac-DEVD-AMC (15 μM) for caspase-3; Ac-VEID-AMC (50 μM) for caspase-6 and Ac-IETD-AMC (15 μM) for caspase-8 like activity measurements. In parallel the lysates were pre-incubated with the respective specific inhibitors Ac-DEVD-CHO (2 μM, caspase-3), Ac-VEID-CHO (1 μM, caspase-6) or Ac-IETD-CHO (0.5 μM, caspase-8) followed by the addition of the respective substrates, and the difference between the two measurements (inhibited activity) were considered the caspase activity. As indicated by the manufacture's instruction, incubation for about 3 h with 1 μg/ml of staurosporin was used as positive control.

2.9. In vivo *tissue and cell localization of crotamine*

For the *in vivo* assays, mice ($n = 10$) were injected intraperitoneally with the fluorescent-labeled crotamine. The control group received the fluorescent dye alone. Mice were sacrificed 3 h after intraperitoneal injection. The living cells from bone marrow were isolated and observed directly under confocal microscope. Also, brain, skeletal

muscle and liver tissues were dissected and 6–10 μm cryostat sections were prepared using a Tissue-Tek embedding matrix (Sakura, Torrance, CA, USA) and a cryo-microtome (Model CM 1100, Leica, Germany). Microscope slides were mounted in anti-fade and observed under confocal laser scanning microscope (LSM 510, Carl Zeiss, Jena, Germany).

3. Results

3.1. Measurement of cell viability: the effect of crotamine on in vitro proliferation of CHO-K1 cells

Considering that a preferential internalization into actively proliferative cells could be observed for crotamine (Kerkis et al., 2004), we elected a highly proliferative cell, namely CHO-K1 cells, for the analysis of the cytotoxic effects of crotamine.

The use of MTT method demonstrated a concentration-dependent cytotoxic effect of crotamine in CHO-K1 cells. After 24 h incubation, crotamine promoted a concentration-dependent cell death in CHO-K1 cells with an IC_{50} value of 5 μM (Fig. 1). As expected, the cytotoxic effect of crotamine was significantly more effective when the cells were cultivated in the presence of 10% of FBS than with 1% FBS, in which a diminished rate of cell proliferation is observed.

Interesting, the pretreatment with 10 μM E-64, an irreversible inhibitor of lysosomal cysteine cathepsins, completely prevented the cell death of CHO-K1 cells cultivated in the presence of 10% FBS, triggered by the crotamine treatment (Fig. 1). It is worth to mention that treatment of these cells with up to 20 μM of E-64 or with 0.001% of saponin did not show any noticeable cytotoxic effect neither influenced on the cell proliferation rate (data not shown).

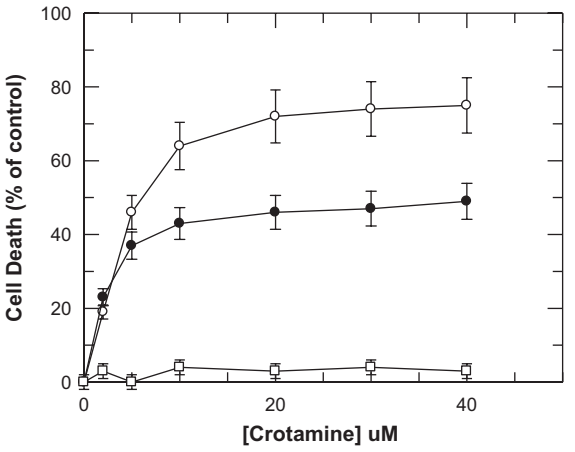

Fig. 1. Cell death of CHO-K1 cells induced by crotamine. CHO-K1 (2×10^4) cells were cultured in the presence of crotamine (0–40 μM) for 24 h in the presence of 100 μl culture media supplemented with 1% (●) or 10% (○) of fetal bovine serum (FBS) for 24 h, at 37 °C, in an atmosphere of 5% CO_2 in air. For the treatment with the E-64 inhibitor, 5 μM of the inhibitor was added 12 h in the presence of 100 μl culture media supplemented with 10% of FBS, before the 24 h treatment with several concentrations of crotamine (□). Percent of cytotoxicity was determined by the MTT viability assay as described in Section 2. Data are expressed as means ± standard deviations of triplicate determinations. The experiment was performed three times in duplicate, with similar results.

3.2. Crotamine accumulation in lysosomes: leakage of lysosomes and cell death in CHO-K1 cells determined by crotamine

In order to compare the crotamine translocation features when used at low and higher concentrations, 0.5 or 5 μM of crotamine conjugated to a fluorescent dye (FITC-crotamine) was added to cultured CHO-K1 cells with the endocytic compartments previously labeled with Lyso Tracker DND-99. This allowed us to immediately observe FITC-crotamine localization within the endosomal/lysosomal vesicles of these pre-labeled unfixed CHO-K1 cells (Fig. 2). FITC-crotamine uptake into endosomal compartment of CHO-K1 cells occurred within less than 5 min (Fig. 2A–F), and its accumulation in the lysosome increased in a time-dependent manner (Fig. 2G–O). It is of note that as early as 15 min after the treatment of CHO-K1 cells with 5 μM FITC-crotamine, it was already possible to observe morphological changes of the cell, such as condensation of chromatin in the nucleus and cell shrinkage (Fig. 2J–O).

Further we monitored the disruption of the lipid bilayer of the acidic endosomal/lysosomal vesicles membrane and subsequent rupture of CHO-K1 cells lysosomes determined by the treatment with higher concentrations of crotamine. For this we have used acridine orange (AO) uptake and relocation methods, as previously described (Olsson et al., 1989; Rundquist et al., 1984; Zdolsek et al., 1999). AO, a metachromatic dye, accumulates mainly in the acidic compartments, preferentially in secondary lysosomes and nucleus. When excited by blue light (488 nm), AO shows different emission wavelengths, red or green fluorescence, depending of the dye concentration. Lysosomal/endosomal compartments accumulates higher concentration of AO, consequently these compartments are labeled in red. In the opposite side, nucleus and cytoplasm accumulates lower dye concentration emitting green light. If, however, merged green and red emission lights are used (uptake method), concentrated and nonconcentrated compartments can be observed simultaneously. Lysosomal AO is observed by its red or orange fluorescence, and nucleus/cytoplasm in green. Rupture of AO-loaded lysosomes can be monitored as an increase in cytoplasmic diffuse green, or a decrease in granular red fluorescence (Brunk and Svensson, 1999; Antunes et al., 2001). Then, previous labeling of lysosomes with AO allows observing an increase in cytoplasmic diffuse orange–yellow or a decrease in granular red fluorescence in response to the rupture of lysosomes (Robbins and Marcus, 1963). Therefore, treatment of cells with crotamine showed an orange–yellow fluorescence, which is representative of an intermediate concentration of AO between red and green fluorescence (Fig. 3). Furthermore, increasing incubation time showed a simultaneous decrease of red fluorescence and increase of green fluorescence, which represents the gradual rupture of the lysosomes, which are organelles containing high concentrations of AO (Fig. 3). Interestingly, the kinetics and concentration-dependence of crotamine accumulation in

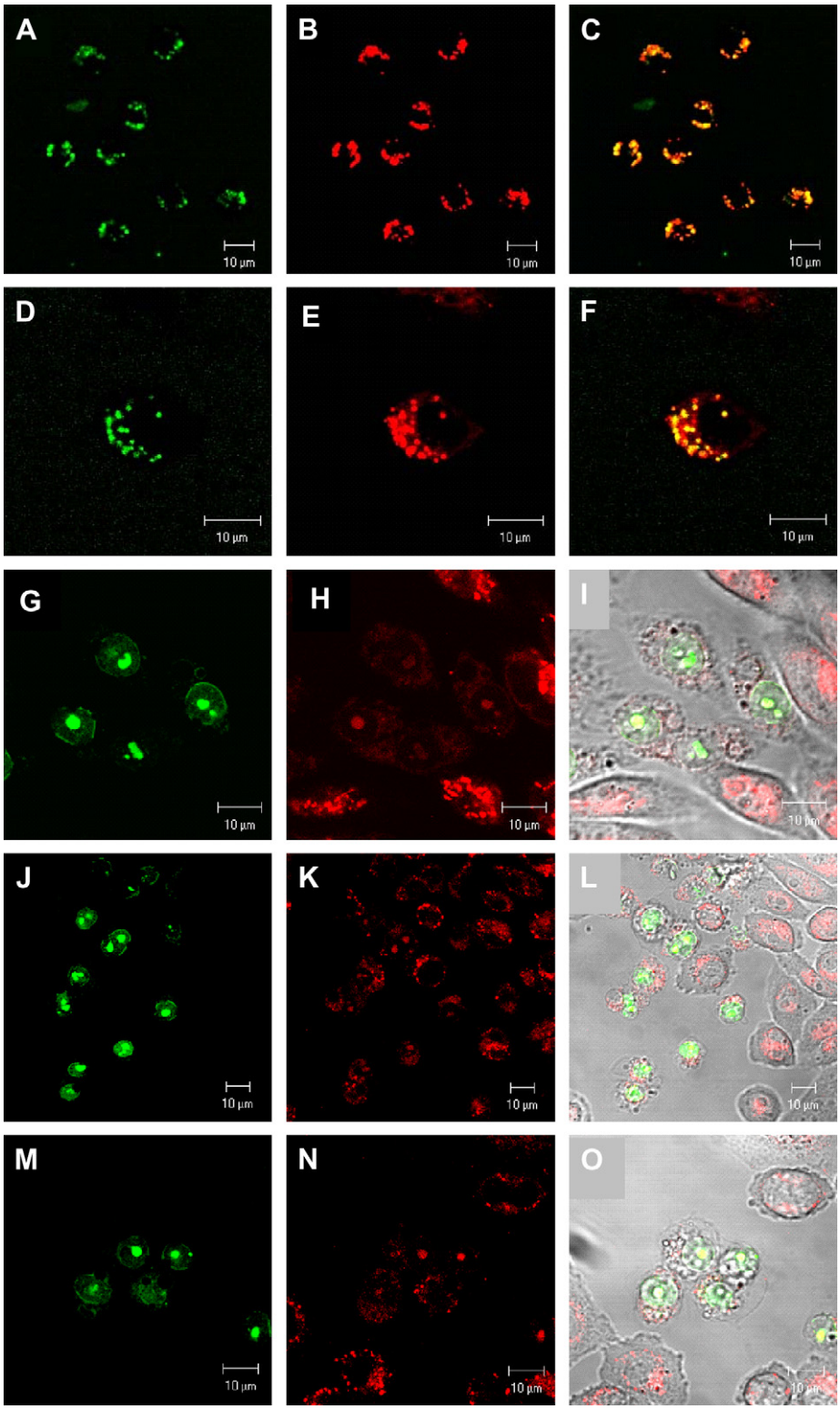

Fig. 2. Immediate uptake of crotamine by CHO cells. The acidic vesicles of endocytic compartments of CHO-K1 living cells were labeled with 1 nM Lyso Tracker DND-99 (red) for 30 min, at 37 °C, followed by incubation with 0.5 μM FITC-crotamine (green) for 5 and 15 min, panels (A–F) and (G–I), respectively; or with 5 μM FITC-crotamine 60 and 150 min (J–L) and (M–O), respectively. Panels (D–F) are magnification of the panels (A–C), respectively. The fluorescent signal of FITC-crotamine (A, D, G, J and M), Lyso Tracker DND-99 (B, E, H, K and N), as well as fluorescent merged images (C and F) and phase DIC micrographs (I, L and O) were monitored at real time with confocal laser scanning microscope. Note the clear morphological changes of the cell in function of high crotamine concentration (5 μM) with longer time incubation (panels J–O), leading to alterations of cell shape and damage of intracellular structures, suggesting a cell death induction and accumulation of FITC-crotamine in the nucleus of the cells. (A–F, G–H, J–K, M–N) = Fcm; (I, L, O) = Fcm + Differential Interference Contrast (DIC); bars = 10 μm.

lysosomes correlate well with their cytotoxic effects in CHO-K1 cells. Morphological changes of the cell, mainly observed as cell shrinkage, were also observed, especially in cells not showing red fluorescence (Fig. 3).

3.3. Crotamine induces lysosome leakage and, consequently, releases lysosomal cysteine proteases into the cytosol and increases caspase-3 activity

As a complement to the immunofluorescence studies, we also monitored the release of lysosomal cysteine proteases into the cytosol after crotamine treatment. In order to assess the effects of crotamine on lysosomal integrity, we monitored the release of cysteine cathepsins over a 3-h time period in cultures permeabilized with 0.001% saponin (Fig. 4). The data presented in Fig. 4 clearly show that crotamine treatment triggered the lysosomal release of cysteine cathepsins to the cell cytosol in CHO-K1 cells. In fact, 5 μM crotamine was able to increase seven-fold the levels of cysteine cathepsins measured in the cell cytosol. In the same way, crotamine treatment was also able to activate caspase-3 (Fig. 5), while no significant activation of caspase-6 and -8 could be observed (data not shown). The caspase-3 activity was increased about 1.5 and 4.5-fold, after crotamine (5 μM) and staurosporin (1 μg/ml) treatment, respectively. As already mentioned, interestingly, the pretreatment of CHO-K1 cells with 10 μM E-64, an irreversible inhibitor of lysosomal cysteine cathepsins, completely prevented the cell death triggered by crotamine treatment (Fig. 1).

3.4. In vivo crotamine uptake into distinct mice tissues and cells

In order to assess the crotamine trafficking within the cells and tissues of adult mice, 1 μM of FITC-crotamine was injected intraperitoneally into mice that were sacrificed 3 h later. Representative data showed that fluorescently labeled crotamine was found in the liver (Fig. 6A and B), bone marrow cells (Fig. 6C and D), in conjunctive tissue adjacent to muscle fibers (Fig. 6E) and brain (Fig. 6F) of injected mice. In brain, strong fluorescence was seen not only in blood vessels, but also within cells with neuron-like morphology. In order to verify the

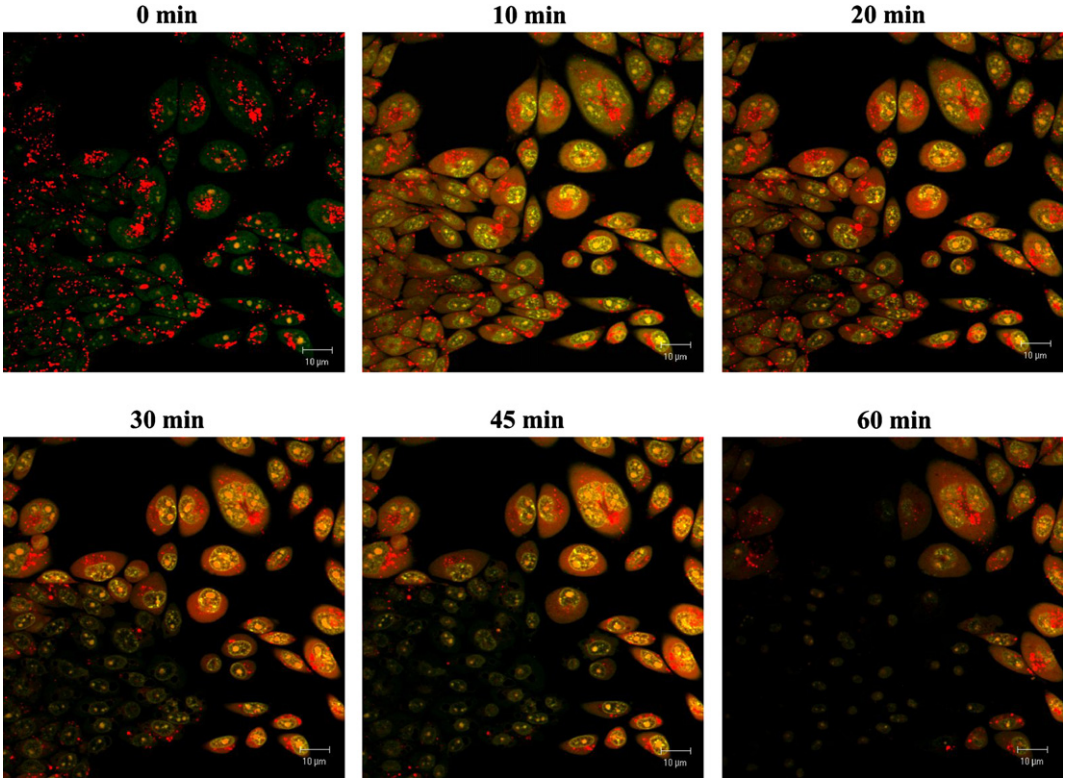

Fig. 3. Lysosomal/endosomal leakage of CHO-K1 cells. Lysosomal/endosomal leakage of CHO-K1 induced by crotamine internalization was monitored using vital acridine orange (AO). For imaging, CHO cells were grown on cover glasses and the living cells were labeled with 5 μg/ml of AO in F-12 medium without serum, for 15 min, at 37 °C. Thereafter, the cells were washed with F-12, and then exposed to 5 μM crotamine for 0, 10, 20, 30, 45 and 60 min, at 37 °C. The fluorescence images were monitored at real time with confocal laser scanning microscope. When excited with blue light at 480 nm AO shows red and green fluorescence at high (lysosomal) or low (nuclear and cytosolic) concentrations, respectively. Rupture of initially acridine orange-lysosomes may be monitored as an increase in cytoplasmic diffuse green, or a decrease in granular red, fluorescence. The orange–yellow fluorescence is representative of an intermediate concentration of acridine orange between red and green fluorescence, showing a decrease of red fluorescence and an increase of green fluorescence at the same time. Note the clear morphological changes of the cells with longer time of crotamine incubation, leading to alterations of cell shape and damage of intracellular structures, suggesting a cell death induction in function of lysosomal rupture. Blue light excitation-induced AO red endosomal/lysosomal fluorescence merged images were monitored at real time with confocal laser scanning microscope. Bars = 10 μm.

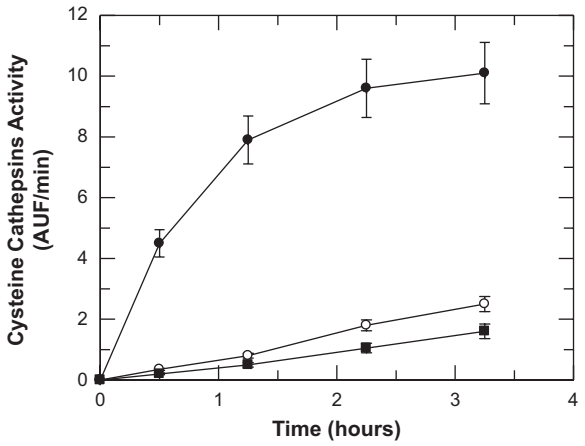

Fig. 4. Crotamine induced lysosomal cysteine cathepsins release in CHO-K1 cells. Culture of CHO-K1 cells were treated with 0.1 (○) or 5 μM crotamine (●) or not (■) for varying lengths of time (0–3 h), at 37 °C. Then the cells were permeabilized with 0.001% saponin followed by collection of extracellular fluids for analyses of cysteine cathepsins activities, as described in Section 2. Data are expressed as means ± standard deviations of triplicate determinations. The experiment was performed three times in duplicate, with similar results.

capacity of crotamine to penetrate into neurons, we incubated Cy3-conjugated crotamine with a primary culture of mouse neurons *in vitro* (see inset on Fig. 6F). Other tissues, such as kidney, spleen and peritoneal liquid cells, also showed significant fluorescent signal indicating the presence of crotamine (data not shown), in the same way as previously shown by using Cy3-crotamine (Kerkis et al., 2004). In control FITC-injected mice, only weak background fluorescence was observed (data not shown).

4. Discussion

Most of toxins are endocytosed before translocation into the cytosol (Chinnapen et al., 2007; Medina-Kauwe, 2007; Broeck et al., 2007; Plaut and Carbonetti, 2008), although in many cases the mechanism by which this occurs has not been fully investigated. In the case of crotamine, we have previously shown that this toxin, at low concentrations, is internalized by endocytosis though binding to heparan sulfate proteoglycans, followed by accumulation into acidic endosomes/lysosomes vesicles (Nascimento et al., 2007). Based on this, we suppose that higher concentrations of crotamine could promote the release of lysosomal hydrolytic enzymes, such as cysteine cathepsins, into the cell cytosol of CHO-K1 cells, triggering a cell death process. In fact, we show here that high concentration of crotamine promotes a large release of lysosomal hydrolytic enzymes, such as cysteine cathepsins, into the cytosol of the CHO-K1 cells, triggering a cell death process (Figs. 1–3).

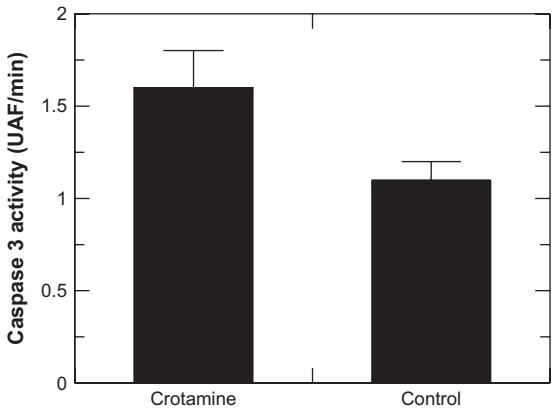

Fig. 5. Crotamine induced caspase-3 activation in CHO-K1 cells. Caspase-3 activation upon crotamine treatment was verified in CHO-K1 cells treated with 5 μM crotamine for 24 h in F-12 medium at 37 °C (*$p < 0.05$). Non-treated cells were used as control. The caspase-3 activity was determined using the caspase-3 fluorometric assay kit, following the manufacturer's instructions as described in Section 2. Data are expressed as means ± standard deviations of triplicate determinations. The experiment was performed three times in duplicate, with similar results.

Interestingly, we could also observe a complete blockage of the cell death triggered by crotamine in the presence of E-64, showing the involvement of endosomal–lysosomal cathepsins in the cytotoxic effects of crotamine. Cathepsins are a group of proteases found predominantly in lysosomes. Cathepsins have enormous destructive potential, and due to this, massive lysosomal rupture was found to lead to necrotic cell death (Bursch, 2001). Moderate lysosomal damage, allowing their release from the lysosomes into the cell cytosol, was also shown to lead to apoptotic cell death (Turk and Stoka, 2007). Involvement of endosomal–lysosomal cathepsins in the apoptosis process has been widely discussed (Leist and Jäättelä, 2001; Salvesen, 2001; Johansson et al., 2003; Stoka et al., 2007), also suggesting the potential advantages of including cathepsin inhibitors in antitumoral therapeutic regimen (Ishisaka et al., 2002; Castino et al., 2002). Fehrenbacher et al. (2004) have shown that deficiency of lysosomal cysteine protease cathepsin B diminished the susceptibility to tumor necrosis factor (TNF)-mediated cytotoxicity increased by immortalization and transformation of murine embryonic fibroblasts. However, it has been suggested that apoptotic cell death induced by cysteine cathepsins is predominantly caspase dependent (Turk and Stoka, 2007).

In fact, our data also shows that crotamine is able to activate caspase-3 (Fig. 5), which is an enzyme capable of disassembling the cell. Active caspase-3 in cells and tissues can execute apoptosis induced by a wide variety of apoptotic signals (Mazumder et al., 2008). It is well-known that lysosomal membrane permeabilization, leading to a leakage of lysosomal cysteine protease(s) into the cytosolic compartment, promotes the activation of caspases and induction of apoptosis (Ishisaka et al., 1998; Erdal et al., 2005). Interestingly, this mechanism is very similar to that recently described for cobra venom cytotoxins, which was first suggested to stimulate the depolarization of myocytes (Harvey, 1985; Hodges et al., 1987) and, more recently, to primary target lysosomes (Feofanov et al., 2005). Even the co-localization of crotamine with Lyso Tracker DND-99 (Fig. 2), the vital fluorescent probe of lysosomes, has a very similar pattern to that described for the cytotoxins from cobra venom, which seems to primary target lysosomes before promoting damage to the plasma membrane (Feofanov et al., 2005).

On the basis of these experimental data presented here, we propose lysosomes as a primary target for the action of

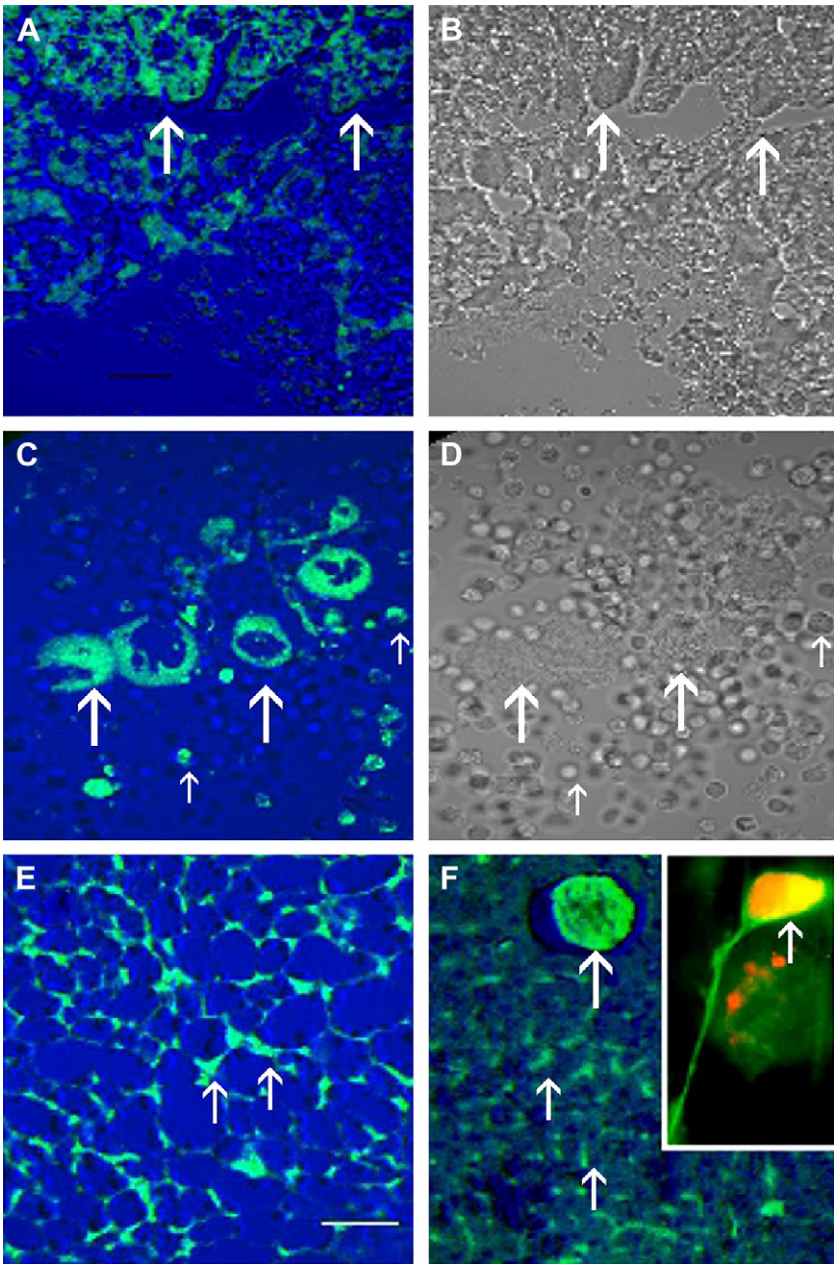

Fig. 6. *In vivo* uptake of crotamine in different cell types. *In vivo* FITC-crotamine uptake observed 3 h after intraperitoneal injection showed fluorescence signals in liver (A and B), bone marrow cells (C and D), skeletal muscle (E) and brain (F) of mice. Fluorescent signal revealed the presence of crotamine in sinusoidal endothelial cells and in the central vein areas of liver lobule (A and B). FITC-crotamine fluorescence was also observed in cytoplasm of mouse megakaryocytes (larger arrows) and other cells (small arrows) from the mouse bone marrow (C and D), and in conjunctive tissue adjacent to muscle fibers (E). In brain, strong fluorescence of FITC-crotamine was seen in neuron-like cells (small arrows) and in blood vessels (indicated by the larger arrow in panel F). Inset on panel (F) show *in vitro* immunostaining of a neuron cell with anti-tubulin III (green) and a Cy3-conjugated crotamine (red) fluorescent labeling of the cell cytosol. The neuron-like cell was immunostained with anti-tubulin III to observe the morphology of the cell (green) and, so forth, the Cy3-crotamine (red) was used this time. The inset shows the nuclear localization of the Cy3-crotamine. (A, C, E, F) Overlay of Fluorescent Confocal Microscopy (Fcm) and Differential Interference Contrast (DIC); (B and D) DIC; F-epi-fluorescence, bars = 10 μm.

crotamine. We have previously shown that, to normal cells, crotamine is non-toxic at the concentrations up to 1.0 μM (Kerkis et al., 2004). Accordingly, we show here that crotamine, only at higher concentrations, i.e., at least above 1 μM, is able to induce appreciable cell death of transformed tumorigenic CHO-K1 cells by increasing lysosomal membrane permeabilization (Figs. 1–3). It is of note that both immortalization and transformation were shown to increase the susceptibility of cells to lysosomal-mediated cell death pathways (Fehrenbacher and Jäättelä, 2005; Erdal et al., 2005).

It is well-known that endocyted basic polypeptides are able to escape from endosomes and to reach the cell cytosol.

The escape from these vesicles is the rate-limiting step for the cytosolic entry of peptides. The endosomal escape seems to be governed by both the concentration of endocyted peptides and the transmembrane pH gradient (inside acidic) across phospholipid bilayers (Magzoub et al., 2005). The crotamine uptake-induced permeabilization of the endosomal/lysosomal membranes might then result in the leakage of the vesicles contents to the cytosol, in the same way as also described for other cationic peptides (Fuchs and Raines, 2004; Nascimento et al., 2007). Indeed, our data show that crotamine is capable of disrupting the lipid bilayer of acidic endosomal vesicles membrane (Fig. 3).

In summary, the experimental data presented here show that crotamine accumulates in lysosomes of CHO-K1 cells in concentration- and time-dependent manner. Moreover, the heavy accumulation of crotamine inside lysosomes promotes lysosomal membrane permeabilization with release of lysosomal enzymes, namely cysteine cathepsins, into cell cytosol and also promotes caspase activation, triggering the cell death process. So forth, it is plausible to infer that crotamine, at higher concentration, could interfere in the normal function of subcellular structures, especially lysosomes, thus affecting the proliferative activity and leading to a cell death. Interestingly, non-proliferative cells showed very modest crotamine translocation (Kerkis et al., 2004). In accordance with this observation, cytotoxicity evaluation by MTT assay showed a stronger crotamine toxic activity on CHO-K1 cells cultivated with 10% of serum than that observed at lower serum concentration (1% FBS), a condition expected to present higher cell proliferation rate.

In spite of the fact that both kidney and liver have been described as the organs that preferentially accumulate the radiolabeled-crotamine injected intraperitoneally in mice (Boni-Mitake et al., 2006), the distribution of crotamine in other tissue as well as at the cellular level has not been thoroughly investigated. Here, we demonstrate the tissue distribution of labeled-crotamine at cellular level after intraperitoneal injection into living mice, which allowed observing crotamine in the cytoplasm of cells from liver, skeletal muscle, brain and kidney (Fig. 6), which are the usual target tissues for the rattlesnakes envenoming process (Azevedo-Marques et al., 1985; Najman and Seshadri, 2007). Moreover, this data is in good agreement with that previously described for distribution of radiolabeled-crotamine (Boni-Mitake et al., 2006).

In vivo fluorescently labeled crotamine administration in living mouse showed here that this toxin is also found in the brain, which suggests it is able to cross the BBB. This observation is in accordance with previous findings from others that have demonstrated that CPPs are able to deliver proteins and other molecules into cells and across the BBB. Three steps for the transcytosis of CPPs through the BBB has been suggested: (1) binding and internalization at the luminal side of endothelial cell membrane, which is negatively charged due to the presence of chondroitin and heparan sulfate residues, through endocytosis; (2) CPP diffusion via the cytoplasm and (3) externalization from endothelial cells (Drin et al., 2002; Stewart et al., 2008).

In conclusion, we showed here that crotamine, either at low or high concentrations (below or above the IC_{50}, respectively), follows the same mechanism of cell penetration previously shown (Nascimento et al., 2007), e.g., through endocytosis, as suggested by the perfect co-localization of FITC-crotamine and Lyso Tracker DND-99 in lysosomes (Fig. 2). However, at higher concentration, crotamine causes CHO-K1 cells lysosome leakage leading to cell death. FITC-crotamine injection into living mice, allowed observing fluorescent signal in the cytoplasm of cells from several tissues of this animal (Fig. 6). This could suggest that crotamine cytotoxic effects might not be restricted to the muscle or brain of the prey, opening the possibility of a role for the crotamine toxic effects also in other tissues. Taken together, our previous (Kerkis et al., 2004; Nascimento et al., 2007) and present data demonstrate that crotamine, when used in different concentrations, shows distinct characteristics that varies from a toxin with cytotoxic properties to a cationic probe and/or a carrier of charged molecules, allowing to propose its use as a biotechnological tool for the delivery of molecules of interest in specific conditions. Both of these finding extend our knowledge about the mechanism of action of toxins in the cells as well as open new perspectives for their application in biomedicine.

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Conflict of interest

The authors declare no conflicts of interest.

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