Taurine chloramine (TN-Cl) is one of the most abundant compounds generated by activated neutrophils. In contrast to HOCl, which causes necrosis, TN-Cl is a potent inducer of apoptosis in tumor cells. Here we show that the apoptosis induced by TN-Cl in human B lymphoma cells is dependent upon oxidant-mediated mitochondrial damage, a decrease in mitochondrial membrane potential, and caspase-9 activation. Further, we show that TN-Cl is taken up into the cells and is concentrated in the mitochondria, where it induces opening of the permeability transition pore (PTP) and mitochondrial swelling. Identical activity is seen upon treatment of isolated mitochondria with TN-Cl and is blocked by the PTP inhibitors bongkrekic acid and cyclosporine A, as well as by the sulphydryl reducing agent TCEP. The data suggest that TN-Cl causes apoptosis through direct damage to the mitochondria.

Neutrophils play an important role in defense against bacterial infections as well as other inflammatory responses. When recruited into the site of inflammation, these cells generate a variety of highly reactive oxidants. Key among these is hypochlorous acid (HOCl), which is produced by the myeloperoxidase-catalyzed oxidation of Cl\(^-\) by H\(_2\)O\(_2\) (1). Hypochlorous acid is a major neutrophil microbicidal and anti-cancer agent but, when produced in excess, it leads to oxidative tissue damage and contributes to the development or progression of disease (2,3). Taurine, a sulphur-containing \(\beta\)-amino acid that is not incorporated into proteins, is the most abundant intracellular amino acid in humans, reaching concentrations of 20-50 mM in leukocytes (4). Stimulated neutrophils release large quantities of taurine that is rapidly chlorinated by the reaction with HOCl, generating taurine chloramine (TN-Cl; \(\text{SO}_3\text{(CH}_2\text{)}_2\text{NHCl}\)) (5,6).

It has been proposed that the presence of taurine and the formation of TN-Cl at inflammatory sites provide a detoxification mechanism for HOCl, providing protection against neutrophil-induced cytotoxicity. TN-Cl is more stable and less toxic than HOCl, but it still retains oxidative potential. Our previous research demonstrated that treatment of human Burkitt’s lymphoma (BL) cells with HOCl in PBS caused rapid necrotic death. In contrast, HOCl treatment in complete cell culture media led to cell death by apoptosis (7). These differences were attributed to the formation of long-lived amino acyl chloramines, formed by the reaction of HOCl with amino acids present in the media. The results could be mimicked by treatment of the cells with taurine chloramine. Thus, conversion of HOCl to TN-Cl may minimize the detrimental effects of an uncontrolled inflammatory response to the necrotic death that would be caused by HOCl alone (2,7-9).

Apoptosis, or programmed cell death, is a complex process that requires the integration of different intracellular signals. It is characterized by a number of well-defined features that include condensation and fragmentation of the chromatin, internucleosomal DNA cleavage, membrane blebbing, caspase activation, translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, and the ultimate formation of so called “apoptotic bodies” (10). Apoptotic cell death, in contrast to necrotic cell death, is thought to be physiologically...
advantageous because the dying cells are cleared by phagocytosis prior to cell lysis and release of potentially inflammatory mediators (11-15). Apoptosis occurs through at least two overlapping pathways referred to as extrinsic and intrinsic. The extrinsic pathway is activated through ligand binding to death receptors (members of the TNF receptor superfamily) at the cell surface, and activation of initiator caspases-8. In the intrinsic pathway, mitochondria play a pivotal early role by releasing cell death signals into the cytosol and activating caspase-9 (16). A common feature of the activation of the intrinsic apoptotic pathway is a sudden increase in the mitochondrial permeability transition (MPT), leading to a decrease in membrane potential (Δψm) and causing mitochondria swelling (17-20). Both pathways converge and activate executioner caspase-3, which then cleaves intracellular protein substrates and causes cell death (21).

It is well recognized that oxidants (e.g. superoxide anion, H2O2, nitric oxide) regulate cell death, but the mechanisms whereby oxidants activate the apoptotic machinery are not well understood and the molecular targets for oxidative modification are not known. The aim of these studies was to better elucidate the pathway involved in TN-Cl-induced apoptosis.

MATERIALS AND METHODS

Cell Culture and Treatments - The Burkitt's lymphoma cell line JLP-119 was grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM L-Gln, and 50 μM β-mercaptoethanol at 37 °C in 5% CO2 in air as described previously (22). Exponentially growing cells were harvested by centrifugation and resuspended in fresh media to achieve a culture density of 0.5 x 10⁶ cells/ml. TN-Cl toxicity studies were carried out by mixing taurine (Sigma) (2 mM in PBS on ice) with NaOCl (Aldrich) (500 μM) and then adding the mixture (1 ml) to cell pellets to get a final cell density of 0.5 x 10⁶ cells/ml. After a 1 h incubation at 37 °C, 1 ml of complete media was added to each well, and cells were incubated for an additional 5 h. TN-Cl preparations made fresh daily and were monitored by ultraviolet absorption spectra (200-300 nm) to confirm the production of the monochloramine as well as the absence of a dichloramine or unreacted NaOCl. Concentrations of stock reagents were based on molar extinction coefficients of 350 M⁻¹ cm⁻¹ for NaOCl at 292 nm, and 429 M⁻¹ cm⁻¹ for TN-Cl at 252 nm (6). Permeable caspase inhibitors (Z-VAD-fmk, Z-IETD-fmk, Z-LEDH-fmk) (B&D Systems), or bongkrekic acid (BA) (Sigma) were pre-incubated with the cells for 1 h. In experiments using thiol reducing agents, cells were pre-incubated with dithiothreitol (DTT) (Sigma) or Tris(2-carboxyethyl)-phosphine (TCEP) (Pierce) for 1 h, washed with PBS and then treated with TN-Cl. None of the inhibitors used in this study had any effect on JLP-119 cell viability at the concentrations used.

Morphological Assessment of Cell Death Using Hoechst 33342/Propidium Iodide (PI) – Treated cells (0.5 x 10⁶ cells/ml) were incubated for 15 min at 37 °C with Hoechst 33342 dye (5 μg/ml in PBS), centrifuged, washed once in PBS, and then resuspended at ~2.5 x 10⁷ cells/ml. PI (Sigma) (50 μg/ml from a 1 mg/ml stock in PBS) was added just before microscopy. Cells were visualized using fluorescence microscopy and a minimum of 200 cells was counted. Cell morphology was classified as follows: (i) live cells (normal nuclei, blue chromatin with organized structure); (ii) apoptotic cells (early apoptotic: bright blue chromatin which is highly condensed, or fragmented; late apoptotic: bright red chromatin, highly condensed or fragmented); (iii) necrotic (red, enlarged nuclei with smooth normal structure) and pyknotic cells (dense, red, slightly condensed nuclei with no fragmentation), as shown in Fig. 1, and described previously (23).

Cellular Uptake, Distribution and Detection of TN-Cl - Treated cells were washed, gently homogenized with a glass-homogenizer in buffer A (220 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, and 0.5 mg/ml fatty acid-free BSA) and sub-fractionated by differential centrifugation into supernatant (cytosol plus membranes) and mitochondria-enriched fractions, as described below and previously (24). Unbroken cells and nuclei were discarded. TN-Cl concentrations were determined by reacting different subcellular fractions with 5-thio-2-nitrobenzoic acid (Nbs)
and measuring absorbance at 412 nm (ε412 nm = 27200 M⁻¹ cm⁻¹), standardized with TN-Cl (ε252 nm = 415 M⁻¹ cm⁻¹) (25). Catalase was added to the Nbs solution to ensure assay specificity. For the radiolabeled TN-Cl uptake experiments, [1,2⁻²H]-TN-Cl was produced by incubating [1,2⁻²H]-taurine (Sigma) with HOCl. Cells were exposed to 100 nM [1,2⁻²H]-TN-Cl in PBS, and incubated at 37 °C for the times indicated in the figure legends. Uptake was stopped by adding an excess of cold taurine on ice. Cells were washed (3x) with PBS + 0.5% BSA, gently homogenized, fractionated by differential centrifugation and the radioactivity in the different subcellular fractions was determined by liquid scintillation counting. Unbroken cells and the nuclear fraction were discarded. The uptake of labeled TN-Cl (fmol/mg protein) was calculated based upon the known specific activity of the radiolabeled TN-Cl solution. Protein concentration was determined by the Bio-Rad protein assay, using BSA as standard. The total [1,2⁻²H]-TN-Cl counts added to the cell suspensions at time zero was 790,386 ± 2,915 cpm, equivalent to 25.9 ± 0.95 pmol (103.6 ± 5.8 nM).

Mitochondrial Permeability Transition (MPT) - Opening of the mitochondrial permeability transition pore (PTP) causes mitochondrial swelling that is measured by the decreased light scattering (and thus absorbance) from suspensions of swollen mitochondria (26). To isolate the mitochondria, cells were gently homogenized using a glass-homogenizer in buffer A and centrifuged at 2,000 g for 10 min at 0 °C to remove nuclei and unbroken cells. Approximately three-quarters of the supernatant was further centrifuged at 10,000 g for 10 min at 0 °C in a new tube. The supernatant of this step is the crude cytosolic fraction, which also contains intracellular and plasma membranes. The fluffy layer of the pellet was removed by gently shaking with buffer A and the firmly packed portion of the pellet was suspended in the same buffer without EGTA and centrifuged at 10,000g for 10 min at 0 °C. The pellet of this centrifugation is the crude mitochondrial fraction. This procedure yielded about 0.7 mg of mitochondrial protein/1x10⁸ cells. Mitochondrial protein was determined by the Bio-Rad protein assay, using BSA as standard. Isolated mitochondria (0.25 mg protein/ml) were suspended in cuvettes in buffer B (210 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH₂PO₄, and 4 µg/ml rotenone). After a 2-min equilibration period, different concentrations of TN-Cl were added, and mitochondrial swelling was followed spectrophotometrically as the decrease in absorbance at 540 nm at 25 °C for 10 min.

Mitochondrial Membrane Potential (Δψm) - Cells (0.5 x 10⁶ cells/ml) were incubated for 15 min at 37 °C with the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetrathiethylbenzimidazolocarbocyanine iodide (JC-1) (Molecular Probes) (5 µg/ml in complete cell culture media), centrifuged, washed once with media, transferred to a 96-well plate (100,000 cells/well), and assayed using a fluorescence plate reader (Molecular Devices) with the following settings: excitation at 485 nm, emission at 540 nm and 590 nm, and cutoff at 530 nm. In some experiments, emission spectra between 530-620 nm were obtained. Δψm was calculated using the ratio of 590 nm (J-aggregates)/540 nm (monomeric form) (27,28).

Confocal microscopy for Δψm - Cells (0.5 x 10⁶ cells/ml) were loaded with JC-1 (5 µg/ml in complete cell culture media), centrifuged, washed once with media, transferred to microscope slides, and examined immediately (28). Samples were viewed at room temperature in a darkened room with a Zeiss LSM Pascal 5 confocal microscope, using 40X/Plan-NeoFluor objective lens. Probes were excited with 488 nm of a 25-mW multi-line argon-krypton laser. JC-1 green fluorescence was visualized with a NFT 545 and BP505-530 emission filter combination. Red fluorescence from JC-1 aggregates was detected using an LP530 emission filter. Photomultiplier settings were the same for all acquisitions.

RESULTS

Apoptosis mediated by TN-Cl involves intracellular oxidation and activation of caspase-9. In order to determine whether TN-Cl activates the extrinsic or intrinsic pathways of
apoptosis, human JLP-119 Burkitt’s lymphoma cells were exposed to 0.5 mM TN-Cl for 6 h. This resulted in 50-60% cell death that was almost entirely early apoptotic, as assessed by nuclear morphology and plasma membrane permeability (Figs. 1 and 2) (23). As shown in Fig. 2A, cell death induced by TN-Cl was prevented by the pan-caspase inhibitor Z-VAD. To determine which initiator caspase was activated in response to TN-Cl, cells were pre-incubated with either the cell-permeable caspase-8 inhibitor Z-IETD, or the caspase-9 inhibitor Z-LEHD. We found that, even at the highest concentration tested (100 μM), inhibition of caspase-8 had no effect on the amount of apoptosis induced by TN-Cl. In contrast, inhibition of caspase-9 caused a dose-dependent inhibition of apoptosis. The activity and specificity of the inhibitors was confirmed in control experiments demonstrating that Z-LEHD inhibited apoptosis induced by staurosporine or VP-16, which are known to induce the intrinsic (caspase-9-dependent) pathway of apoptosis, and Z-IETD inhibited apoptosis induced by anti-Fas antibody, which is known to act through the extrinsic (caspase-8-dependent) pathway (data not shown).

Even though TN-Cl is considered to be a relatively weak oxidant (compared to HOCl), it is known to oxidize biomolecules, especially those containing sulfhydryls and methionine (29,30). To determine whether cell death induced by TN-Cl involves an oxidative mechanism, we compared the effects of two different thiol reducing agents – one that is cell-permeable (TCEP), and one that is cell-impermeable (DTT). Cells were pre-incubated with either reducing agent and then washed prior to TN-Cl treatment. Fig. 2B shows that only TCEP inhibited induction of apoptosis by TN-Cl. Overall, these results suggested that apoptosis induced by TN-Cl is dependent on the oxidation of an intracellular target that activates the mitochondrial cell death pathway.

TN-Cl is taken up by Burkitt’s lymphoma cells and concentrates in the mitochondria. Biological membranes are impermeable to passive transfer of hydrophilic anions such as TN-Cl, and it is thought that TN-Cl has low permeability to cells (6,31). However, TN-Cl is transported into human erythrocytes by the anion-transport system (32), and has been shown to oxidize intracellular IkBα in human T leukemia (Jurkat) cells (33). To test whether TN-Cl is taken up into JLP-119 BL cells, we measured the uptake of radiolabeled TN-Cl, prepared by mixing [1,2-3H]taurine with HOCl. The results (Fig. 3A) show that the cells incorporated TN-Cl at a rate that was similar to the uptake of tritiated taurine ([1,2-3H]-taurine). After 1 h of incubation, the BL cells had incorporated 3% of the total [1,2-3H]-TN-Cl added. Sub-cellular fractionation experiments indicated that labeled TN-Cl and taurine were preferentially taken up into the mitochondria-enriched fraction as compared to the crude cytosolic fraction (Fig. 3B). This finding was confirmed by an orthogonal method using the colorimetric sulfhydryl compound 5-thio-2-nitrobenzoic acid (Nbs) to detect chloramines. Cells were treated with 0.5 mM TN-Cl for 1 h in PBS. N–Cl derivatives rapidly oxidize the sulfhydryl group in Nbs to form a colorless disulfide (Nbs2). Only the mitochondria-enriched fraction contained detectable chloramines (26.1 ± 8.9 μM of TN-Cl/mg of mitochondrial protein), confirming the results obtained with radiolabeled TN-Cl.

TN-Cl induces mitochondrial swelling that is dependent on opening of the permeability transition pore (PTP) complex. In mitochondria-driven apoptosis, the mitochondria undergo a permeability transition (MPT; reviewed in Refs. 18,20). The MPT refers to the swelling and depolarization of the mitochondria that occurs under some conditions, most notably as a result of calcium overload and oxidative stress (34). Studies were carried out using isolated mitochondria to determine whether TN-Cl can induce mitochondrial swelling directly. As shown in Fig. 4A, TN-Cl induced MPT in the mitochondria in a dose-dependent manner. In contrast, even at the highest dose tested (800 μM), taurine did not induce mitochondrial swelling (data not shown), arguing against a non-specific, osmotic effect of TN-Cl.

The cause of MPT is the opening of a non-specific pore in the inner mitochondrial membrane, known as the mitochondrial permeability transition pore (PTP) complex. We found that the specific PTP inhibitors cyclosporine A (CsA) and bongkrekic acid (BA)
inhibited TN-Cl-induced mitochondrial swelling (Fig. 4B). The sulfhydryl reducing reagent TCEP also inhibited the direct induction of mitochondrial swelling by TN-Cl (Fig. 4C). The data suggest that TN-Cl directly induces PTP-dependent swelling of isolated mitochondria and likely acts through thiol oxidation.

To confirm the involvement of PTP opening in TN-Cl-induced cell death, we tested the effect of the PTP inhibitor bongkrekic acid on the induction of apoptosis caused by TN-Cl in intact BL cells (Fig. 5). Bongkrekic acid was effective at inhibiting apoptosis at concentrations of 10-50 μM. In contrast, CsA was ineffective at inhibiting TN-Cl-induced apoptosis (data not shown).

TN-Cl induces loss of mitochondrial membrane potential (Δψm) that precedes apoptosis. PTP opening results in the re-equilibration of solutes of up to ~1.5 kDa and causes mitochondrial swelling, with a consequent decrease in Δψm and the release of apoptogenic signals into the cytosol (20). Confocal analysis of BL cells stained with the cationic carbocyanine dye JC-1 showed that JC-1 localized to the mitochondria of untreated cells, where it formed red fluorescent J-aggregates, typical of high mitochondrial membrane potential, and a punctate intracellular pattern (Fig. 6A). Following exposure to 200 nM valinomycin, a K⁺ ionophor that induces Δψm collapse, the dye formed the expected green fluorescent monomers and partitioned into the cytoplasm (Fig. 6B). A similar but less complete molecular shift was observed following treatment of the cells with 0.5 mM TN-Cl for 6 h. To determine if the loss of Δψm was an early or late event in the apoptosis induced by TN-Cl, we performed a time course experiment and followed fluorometrically the red/green fluorescence emission intensity in JC-1-stained cells. Both valinomycin (positive control) and TN-Cl caused a green shift in the JC-1 emission spectrum, which is characteristic of dye monomerization (Fig. 7A). Valinomycin caused a total and irreversible collapse in Δψm within 5 min of treatment (time course not shown). TN-Cl acted more slowly and caused a continuous decrease in Δψm that was first detected in the first 30 min of incubation (Fig. 7B). Fig. 7C shows that the loss of Δψm preceded the appearance of the morphological markers used to quantify apoptosis (nuclear condensation and fragmentation).

DISCUSSION

The novel findings presented here are that TN-Cl causes direct mitochondrial oxidative damage and PTP-dependent swelling, leading to caspase-9-dependent and caspase-8-independent apoptosis. Thus, TN-Cl caused a decrease in Δψm (JC-1 monomerization) in intact cells and swelling of isolated mitochondria, both of which reflect PTP function. The swelling of isolated mitochondria was inhibited by pre-incubation with the thiol antioxidant TCEP and by two blockers of the PTP (bongkrekic acid and cyclosporine A). Induction of apoptosis by TN-Cl was also inhibited by bongkrekic acid and TCEP, as well as by an inhibitor of caspase-9 but not by an inhibitor of caspase-8. The data support the hypothesis that TN-Cl causes apoptosis by targeting the mitochondria and causing oxidant-dependent PTP opening. These results will be discussed further below.

Mitochondrial (dys)function in response to oxidative stress is a central event leading to cell death (18,34-36). Even though lymphoid cells have relatively small numbers of mitochondria (8 to 15 per cell) compared to the thousands found in liver cells (37), they are crucial for the production of metabolic energy in these cells and also play a key role in controlling the pathways that lead to cell death (38). This was true for the mitochondrial population in JLP-119 cells as well, based on JC-1 staining and confocal microscopy. We found that TN-Cl was concentrated in the mitochondria, and treatment of isolated mitochondria with TN-Cl caused irreversible organelle swelling. Moreover, we tried but were unable to generate a BL cell line depleted of mitochondrial DNA (JLP-119 ρ⁰ cells) using a protocol described elsewhere (39). After the first 10 days in culture, all cells had died (data not shown), consistent with the importance of the mitochondria for viability in this cell type.

The ability of TN-Cl to cause mitochondrial damage (swelling and
depolarization) was inhibited by a thiol antioxidant, indicating that the TN-Cl acts through an oxidative mechanism. The finding that blockers of the PTP prevented TN-Cl-induced mitochondrial damage points to the PTP or associated proteins as being the target of TN-Cl activity. Protein sulfur groups (cysteine sulfhydryls and methionine thioethers) are known to be primary targets of TN-Cl oxidation (29). Thiols with a low pK and that have a high positive charge in close proximity are particularly susceptible to oxidation by TN-Cl. It is thought that the MPT is modulated by the redox status of mitochondria (i.e. glutathione, thioredoxin, and NAD(P)H levels) (34,36), and recent data suggest that the adenine nucleotide translocator component of the PTP is highly sensitive to oxidative stress (35,36), with three cysteine residues, Cys^56, Cys^159, and Cys^256 showing differential reactivity towards thiol oxidizing agents (17). These cysteines all face the mitochondrial matrix and may be the thiol groups that regulate PTP opening and mitochondrial swelling. Although a wide variety of antioxidants have been shown to protect the mitochondria, the finding that a thiol peroxidase protects against MPT is especially significant since it indicates that MPT involves oxidation of pore complex-associated sulfhydryls (40). Future studies will characterize the molecular events associated with TN-Cl oxidation of the permeability transition pore complex.

There is some controversy in the scientific literature over whether TN-Cl is taken up into mammalian cells. The passive membrane permeability of TN-Cl is believed to be low, but active transport of TN-Cl into cells has been described for erythrocytes (32) and RAW 264.7 cells (41). Oxidation of intracellular IkBα by TN-Cl further supports the conclusion that TN-Cl enters cells (33). On the other hand, it has been argued that TN-Cl is not taken up into cells and that alternative mechanisms account for TN-Cl-induced cellular effects. Peskin et al. (42) recently demonstrated that TN-Cl exchanges its oxidized Cl\(^-\) group with other amines and amino acids (e.g. glycine) in the medium, generating chloramines that are more cell permeable (e.g., Gly-Cl\(^-\)). The authors concluded that this exchange is responsible for the intracellular effects of TN-Cl. However, our experiments showing incorporation of radioactivity from tritium-labeled TN-Cl were carried out in buffer lacking other amines, thus avoiding a possible chloramines exchange mechanism. It might be argued that, since the tritium label is on the taurine moiety, what we were actually measuring was the uptake of free taurine released after interaction of TN-Cl with cell surface molecules. This mechanism has not been formally excluded, but free taurine is not toxic, and does not damage the mitochondria (Fig. 4), so it cannot account for the effects of TN-Cl. Moreover, if TN-Cl were acting at the cell surface, it would be expected to require activation of caspase-8 to induce apoptosis, and should have been preventable by both cell-permeable and -impermeable reducing agents, but this was not the case. Still, the molecular mechanism whereby TN-Cl is transported into the lymphoma cells is unknown and needs to be explored further. Since the BL cells incorporated TN-Cl at the same rate as taurine, we would hypothesize that both compounds employ the same cellular import machinery. Interestingly, pretreatment of the cells with CsA, a classical MPT blocker, enhanced the total amount of cell death induced by TN-Cl (data not shown), while it inhibited the MPT and swelling in isolated mitochondria. CsA is known to inhibit the P-glycoprotein present in the plasma membrane of tumor cells (43), and thereby inhibits the active efflux of several classes of chemotherapeutic compounds. Some taurine-related compounds have been described as P-glycoprotein substrates (44), suggesting that cyclosporine A may have inhibited the efflux of TN-Cl as well.

The mechanisms by which oxidants regulate biological processes have been the subject of much research. It is clear that excess levels of free radicals can cause cell death, but the mode of death (i.e. necrotic, apoptotic, other) depends on the type, concentration, source and environment of the oxidant(s) (7). Our research and that of others shows that TN-Cl and other amino acyl chloramines causes apoptosis in a variety of cell types (7,45-48) and it does so at concentrations that are biologically relevant. The physiological concentrations of TN-Cl have not been determined directly, but in vitro data show that in the presence of a physiologically
relevant (~15 mM) concentration of taurine, activation of human neutrophils (~2 x 10^6 cell/ml) results in generation of ~100 µM TN-Cl (6). Taking into account that an inflammatory site is heavily infiltrated with neutrophils, it is conceivable that local accumulation of TN-Cl may reach the mM range. The high physiological concentrations of taurine in cells and tissues scavenge neutrophil-derived HOCl, which, by itself, causes solely necrotic cell death (7). Hence, the formation of TN-Cl switches cell death from necrosis, which can cause more extensive tissue damage, to apoptosis, which is generally believed to be more physiologically beneficial (2,10-13).

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**FOOTNOTES**

* We thank Dr. Salvatore Alesci for expert assistance with the taurine uptake experiments, and David Goucher for expert assistance with confocal microscopy. F. K. was supported by a Training Grant from ORISE.

1 Abbreviations used in the text are as follows: TN-Cl, taurine chloramine; PTP, permeability transition pore; MPT, mitochondrial permeability transition; PI, propidium iodide; BA, bongkrekic acid; CsA, cyclosporine A; TCEP, Tris(2-carboxyethyl)-phosphine; Nbs, 5-thio-2-nitrobenzoic acid; DTT, dithiothreitol; JC-1, 5,5’,6,6’-tetrachloro-1’,3’,3’-tetraethylbenzimidazolcarbocyanine iodide; BSA, bovine serum albumin.
FIGURE LEGENDS

Fig. 1. Morphological assessment of JLP-119 cell death by Hoechst 33342/propidium iodide (PI) nuclear staining.

Fig. 2. Effect of caspase inhibitors and thiol reducing agents on cell death induced by TN-Cl. JLP-119 cells (0.5 x 10^6/ml) were pretreated for 1 h with different concentrations of caspase inhibitors (A) or thiol reducing agents (B) in complete media and then exposed to 0.5 mM TN-Cl in PBS. After 1 h at 37 °C, complete medium was added and the cells were incubated for an additional 5 h. Cell death was morphologically assessed by Hoechst 33342/PI double staining and classified as shown in Fig. 1 and as described in Material and Methods. For thiol reducing agent experiments, cells were washed before TN-Cl treatment. The data represent averages from at least 3 independent experiments carried out in triplicate.

Fig. 3. Uptake of labeled TN-Cl by JLP-119 cells. Cells (1 x 10^6/ml) were incubated with 100 nM [1,2-^3H]-TN-Cl (O) or [1,2-^3H]-Tau (●) at 37 °C in PBS, washed, and the radioactivity measured at the indicated times. (A) total cellular uptake, expressed as fmol/1 x 10^6 cells, (B) sub-cellular distribution measured in crude mitochondrial (dashed line) and cytosolic (supernatant, solid line) fractions. The data represent means ± S.E. of two independent experiments carried out in triplicate.

Fig. 4. Permeability transition (MPT) in isolated mitochondria treated with TN-Cl. Mitochondria (0.25 mg/ml) were isolated from JLP-119 cells and exposed (arrows) to different combinations of treatments. Mitochondrial swelling (light scatter at 540 nm) was used as a measure of MPT, as described in Materials and Methods. (A) Dose response to TN-Cl (0-200 µM). (B) Effect of the PTP inhibitors bongkrekic acid (BA) (50 µM) and CsA (2 µM) on swelling induced by 100 µM TN-Cl. (C) Effect of the sulphydryl reducing agent: TCEP (100 µM) on swelling induced by 100 µM TN-Cl. The data represent means of 3 to 5 independent experiments carried out in triplicate.

Fig. 5. Effect of a mitochondria permeability transition pore inhibitor on cell death induced by TN-Cl. Cells (0.5 x 10^6/ml) were pre-treated with different concentrations of bongkrekic acid (BA) for 1 h in complete media and then exposed to 0.5 mM TN-Cl in PBS. After 1 h at 37 °C, complete medium was added and cells were incubated for additional 5 h. Cells were washed and stained with Hoechst 33342/PI to determine cell death by nuclear morphology, as described in Material and Methods and as shown in Fig. 1. The data represent averages from at least 3 independent experiments carried out in triplicate.

Fig. 6. Confocal microscopy analysis of mitochondrial membrane potential (ΔΨm) in JLP-119 cells treated with TN-Cl. Cells (0.5 x 10^6/ml) were treated with 0.5 mM TN-Cl for 6 h at 37 °C, washed, and incubated, in media, with JC-1 (5 µg/ml) at 37 °C. After 15 min, cells were washed and analyzed immediately. As a positive control, cells were treated with valinomycin (200 nM). The images (400X magnification) are representative of 3 independent experiments carried out in duplicate. Inner boxes: single cells in detail.

Fig. 7. Time course of the loss of ΔΨm and cell death in JLP-119 cells treated with TN-Cl. Cells (0.5 x 10^6/ml) were treated with 0.5 mM TN-Cl, washed, and incubated with JC-1 (5 µg/ml) at 37 °C for 15 min. (A) The JC-1 emission spectra (530-620 nm) were taken on untreated, valinomycin- (200 nM), and TN-Cl-treated cells (0.5 mM) (6 h of treatment shown). (B) The ratio between the emission intensities at 590/540 nm gives a measure of ΔΨm at the indicated times in untreated cells (●) and TN-Cl-treated cells (O). (C) Comparison of the loss of ΔΨm (dashed line) and cell death (solid line) in TN-Cl-treated (O) and untreated cells (●). Cell death was assessed by nuclear morphology in cells stained with Hoechst/PI,
using fluorescence microscopy. The data represent means ± S.E. for at least 3 independent experiments carried out in triplicate.
Figure 1

Live (viable)

Apoptotic
- Early
- Late

Pyknotic

Necrotic
Figure 2

A  Caspase inhibitors

B  Reducing agents
Figure 3

A

![Graph A]

B

![Graph B]
Figure 4

(A) Figure showing the change in absorbance (A₅₄₀) over time (200 sec) for different concentrations of PTP inhibitors: 0, 10 μM, 25, 50, 100, and 200.

(B) Graph showing the effect of PTP inhibitors on swelling. The graph compares untreated and treated samples with BA or CsA.

(C) Graph showing the effect of -SH reducing agent on swelling. The graph compares untreated and treated samples with TCEP.
Figure 5

![Bar graph showing cell death percentage](image)

- Untreated
- TNCl 500uM
- TNCl + BA 10uM
- TNCl + BA 25uM
- TNCl + BA 50uM

Legend:
- ■ pyknosis/necrosis
- □ apoptosis
Figure 6

A  untreated

B  valinomycin

C  TNCI
Figure 7

A
untreated
valinomycin
TNCI

B
incubation time (min)
Abs 590/540
untreated
TNCI

C
incubation time (h)
(% of control cells)
untreated (Δpsi)
TNCI (Δpsi)
untreated (viability)
TNCI (viability)
Taurine chloramine, an oxidant derived from neutrophils, induces apoptosis in human B Lymphoma cells through mitochondrial damage
Fabio Klamt and Emily Shacter

J. Biol. Chem. published online March 30, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M501170200

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