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Superoxide anions mediate veratridine-induced cytochrome *c* release and caspase activity in bovine chromaffin cells

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1 Mitochondrial mechanisms involved in veratridine-induced chromaffin cell death have been explored.

2 Exposure to veratridine (30 μM, 1 h) produces cytochrome *c* release to the cytoplasm that seems to be mediated by superoxide anions and that is blocked by cyclosporin A (10 μM), MnTBAP (10 nM), catalase (100 IU ml^{-1}) and vitamin E (50 μM).

3 Following veratridine treatment, there is an increase in caspase-like activity, blocked by vitamin E (50 μM) and the mitochondrial permeability transition pore blocker cyclosporin A (10 μM).

4 Superoxide anions open the mitochondrial permeability transition pore in isolated mitochondria, an effect that is blocked by vitamin E (50 μM) and cyclosporin A (10 μM), but not by the Ca^{2+} uniporter blocker ruthenium red (5 μM).

5 These results strongly suggest that under the stress situation caused by veratridine, superoxide anions become important regulators of mitochondrial function in chromaffin cells.

6 Exposure of isolated bovine chromaffin mitochondria to Ca^{2+} results in mitochondrial swelling. This effect was prevented by ruthenium red (5 μM) and cyclosporin A (10 μM), while it was not modified by vitamin E (50 μM).

7 Veratridine (30 μM, 1 h) markedly decreased total glutathione and GSH content in bovine chromaffin cells.

8 In conclusion, superoxide anions seem to mediate veratridine-induced cytochrome *c* release, decrease in total glutathione, caspase activation and cell death in bovine chromaffin cells.
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Keywords: Chromaffin cells; veratridine; superoxide; mitochondria; caspases; permeability transition pore

Abbreviations: $\Delta\Psi$m, mitochondrial potential; AFC, 7 amino-4 trifluoromethyl-coumaryl; AFU, arbitrary fluorescent units; BSO, buthionine sulfoximine; $[Ca^{2+}]_i$, intracellular free calcium concentration; CsA, Cyclosporin A; cyt *c*, cytochrome *c*; DEVD-AFC, Asp-Glu-Val-Asp-7 amino-4 trifluoromethyl-coumaryl; DMSO, dimethyl sulphoxide; DMEM, Dulbecco's modified Eagle's medium; DNTB, 5,5′-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; GSt, total glutathione; Het, Hydroethidine; K-H, Krebs HEPES buffer; MnTBAP, Mn(III) tetrakis (4-benzoic acid) porphyrin; ·OH, hydroxyl radical; PBS, Phosphate buffered saline; PTP, permeability transition pore; ROS, reactive oxygen species; RR, ruthenium red; SSA, 5-sulphosalicilic acid; SOD, superoxide dismutase; TNB, 5-thio-2 nitrobenzoic acid; TTX, tetrodotoxin; Vit E, vitamin E; XO/X, xanthine oxidase/xanthine

Introduction

Veratridine prevents inactivation of voltage-dependent Na^+ channels (Catterall & Coppersmith, 1981) keeping them open for long periods of time (Bönisch & Keller, 1983) and producing an increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ (Amy & Kirshner, 1982). As a consequence of these changes, veratridine is able to produce Ca^{2+}-dependent death in excitable cells including rat neurons (Pauwels *et al*., 1989) and bovine chromaffin cells. In the latter case, the toxic effect of veratridine was mediated by superoxide (O_2^-) anion production and caspase activation (Jordan *et al*., 2000).

Superoxide anions result from a single electron acceptance by an O_2 molecule. Its intracellular sources include mono-oxygenases and NADH dehydrogenase, cyclo-oxygenase, lipoxygenase, xanthine oxidase and some components of the electron transport chain (Fridovich, 1997). Superoxide anions may pass through membranes *via* anion channels and act as an electrophile or nucleophile, and potentially forms more reactive species *via* secondary reactions to form H_2O_2 and secondary radicals such as ·OH generated *via* the Fenton reaction. Excessive biosynthesis of O_2^- varies with metabolic activity and age and its generation increases after treatment with neurotoxic drugs as NMDA (Bindokas *et al*., 1996),

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MPP^+ or veratridine (Jordan *et al*., 2000). In this context, over expression of superoxide dismutase using adenoviral vectors or increasing superoxide scavenging capacity of the cells protect against cytotoxic stimuli like growth factor deprivation or exposure to neurotoxins (Jordan *et al*., 1995; Prehn *et al*., 1997; Estévez *et al*., 1998).

Mitochondria are the main source of O_2^- in the cells and an increasing body of evidence suggests that this organelle appears to be able to participate in cellular pathology through activation of a channel found in the inner membrane called mitochondrial permeability transition pore (PTP) (Hirsch *et al*., 1998; Miller, 1998). Pore opening is favoured by several factors including depolarization, increases in intramatrix Ca^{2+} and oxidizing agents and, under some conditions, PTP activation appears to be irreversible and has profound consequences for cell function, being associated with mitochondrial swelling and release of molecules into the cytoplasm, like apoptosis-inducing factor and cytochrome *c* (cyt *c*), which appear to be an early event in the triggering of death in many cell types, modulating the execution phase of apoptosis, through activation of proteases like caspases (Kroemer *et al*., 1997; Bernardi *et al*., 1999).

In the present work, we have explored the role of O_2^- as one of the signals mediating veratridine-induced death in chromaffin cells and found that O_2^- is able to open mitochondrial PTP and also plays a role in caspase activation in response to veratridine. In addition, we have observed that veratridine induces release of cyt *c* from mitochondria and that this action is antagonized by vitamin E, one of the major lipophilic antioxidant agents and reproduced by the O_2^- generating agent paraquat. These results further support the use of bovine chromaffin cells as a good model to study molecular mechanisms involved in neural death.

Methods

Chromaffin cell culture

Bovine chromaffin cells were isolated as previously described (Calvo *et al*., 1995). After washing with a Ca^{2+}-free Locke's solution (Locke medium) containing (in millimoles L^{-1}: NaCl 154, KCl 5.6, $MgCl_2$ 1, HEPES 10, Glucose 10, pH 7 to remove remaining erythrocytes, adrenal glands were then incubated with Ca^{2+}-free Locke's medium containing 0.2% collagenase (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) and 0.5% bovine serum albumin (Calbiochem, La Jolla, CA, U.S.A.) for 45 (3×15) min. Following medulla dissection and further incubation in collagenase solution for 30 additional minutes, chromaffin cells were separated from erythrocytes using a Percoll gradient and plated (2×10^6 cells ml^{-1} in culture flasks), in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, penicillin (100 IU ml^{-1}) and streptomycin (50 μg ml^{-1}) at 37°C under an atmosphere of 5% CO_2.

Assay of caspase enzymatic activity

Six hours after treatment with 30 μM veratridine, bovine chromaffin cells were collected and lysed in a buffer with the following composition (in millimoles L^{-1}): HEPES 25; EDTA 5; EGTA 1; $MgCl_2$ 5; DTT 5; PMSF 1 and 10 μg ml^{-1} each of pepstatin and leupeptin; pH 7.5. Caspase activity was measured as previously described using the fluorogenic substrate Asp-Glu-Val-Asp-7 amino-4 trifluoromethyl-coumaryl (DEVD-AFC) (15 μM in DMSO, Calbiochem System Products) (Jordan *et al*., 2000). Enzymatic activity was expressed as nmoles of AFC releasing/mg protein/min using AFC as standard. Vitamin E (50 μM) and CsA (10 μM) were added 1 h before and maintained during veratridine (30 μM; 1 h) treatment.

Mitochondria isolation

Mitochondria were isolated from bovine adrenal medulla essentially as previously described (Campo *et al*., 1992). Briefly, tissue was manually homogenized with four strokes with a Teflon pestle in solution I (Sol I) containing (in millimoles L^{-1}) mannitol, 230; sucrose, 70; EGTA, 1 and HEPES, 5, pH 7.4 on ice. After centrifugation at 4°C ($106 \times g$; 80 s), supernatant was layered onto solution II containing (in millimoles L^{-1}): mannitol , 460; sucrose, 14; EGTA, 1 and HEPES, 10; pH 7.4), and centrifuged ($800 \times g$; 3 min). The top layer was then centrifuged ($2000 \times g$; 5 min) and the mitochondrial pellet resuspended in (mM): mannitol 215, sucrose 71, succinate 10 and HEPES 10, pH 7.4 and kept on ice until PTP determinations.

Permeability transition pore activity

Permeability transition pore opening was assayed spectrophotometrically as previously described (Kristal *et al*., 2000). Specifically, mitochondria were suspended in 200 μl of a solution containing (mM): mannitol 215, sucrose 71, succinate 10 and HEPES 10, pH 7.4. Changes in absorbance at 540 nm (A_{540}), indicating mitochondrial swelling due to PTP opening, were measured, after addition of different compounds, using a microplate reader (BioRad, Hercules, CA). Initial A_{540} values were $\cong 0.8$, and minor differences in loading were compensated by representing the data as the fraction of the initial absorbance measure remaining at a given time.

In vitro *superoxide production*

As superoxide generator system we used an enzymatic model based in xanthine oxidation by xanthine oxidase (XO/X), by mixing fresh solutions of xanthine (2 mM final concentration) and xanthine oxidase (2 u ml^{-1}, salicylate-free, from bovine milk, specific activity 1 u mg^{-1} of protein) in 0.150 M NaCl-0.01 M sodium phosphate (pH 7.4), as described previously (Napoli *et al*., 2000).

As a control, to analyse that the observed effects are most likely due to superoxide anions and not to degradation products, we performed incubations with XO/X solution made 24 h in advance containing only degradation products of XO/X system. This solution was unable to generate O_2^- and did not produce mitochondrial swelling (data not shown).

Fluorometric determinations

Superoxide production was monitored using hydroethidine (HEt, Molecular Probes) as described previously (Jordan *et al*., 2000). Background was subtracted, and fluorescence

recorded using an excitation filter of 535 nm and an emission filter of 635 nm (Omega Optical Inc.; Brattleboro, VT, U.S.A.). Fluorescent images were recorded every 10 s over a 7 min period. Linear regression of fluorescence data was obtained for each condition and the slope of the best fitting line was taken as an index of O_2^- production.

However, one of the limitations of hydroethidine as a tool for quantitation of O_2^- production is the increase in quantum efficiency (about 20 fold) that occurs following ethidium binding to either DNA or RNA (Lepecq & Paoletti, 1967). This action makes that absolute quantitation of O_2^- production requires the presence of both DNA and RNA at the proper ratio and concentrations. For this reason, O_2^- production is generally expressed as arbitrary fluorescence units (AFU).

Cytochrome c *determination*

Immunoblot analysis was performed on cytosolic extracts from control and veratridine-treated cultures, as previously described (Bobba *et al*., 1999). Cells were washed once with PBS and collected by centrifugation ($2000 \times g$; 5 min). Cell pellet was resuspended in 200 μl of extraction buffer containing (mM): sucrose 250, Tris-HCl 50, EGTA 1, EDTA 1, DTT 1, PMSF 0.1, pH 7.4. Cells were homogenized in a Teflon-glass homogenizer (5 strokes) and, after 15 min on ice, the suspension was centrifuged ($15{,}000 \times g$; 15 min). The supernatants, i.e. cytosolic fractions, were removed and stored at -80°C until analysed by gel electrophoresis. Fifteen μg of cytosolic proteins were loaded onto a 15% SDS-polyacrylamide gel, separated and transferred to a PVDF membrane, that was incubated with anti-cytochrome *c* (1 : 1000 dilution of rabbit polyclonal IgG, Santa Cruz Biotechnology Inc.). The signal was detected using an enhanced chemiluminescence detection kit (Amersham ECL RPN 2106 Kit).

Cell viability experiments

Coverslips containing chromaffin cells were rinsed twice with Krebs-HEPES buffer (K-H) with the following ionic composition (in millimoles L^{-1}): NaCl 140; KCl 5.9; $MgCl_2$ 1.2; HEPES 15; glucose 10; $CaCl_2$ 2.5; pH 7.4, incubated for different times either in K-H or in K-H containing the indicated drugs, and exposed for 1 h to veratridine (30 μM) at room temperature. Exposure was terminated by washing the cells three times with K-H solution. Cellular death was determined using fluorescein diacetate/propidium iodide double-staining procedure (Jordan *et al*., 1997). Twenty-four hours after exposure to veratridine, chromaffin cells were incubated for 45 s at 22–25°C with 15 μg ml^{-1} fluorescein diacetate (Sigma) and (4.6 μg ml^{-1}) propidium iodide (Molecular Probes, Inc. Eugene, OR, U.S.A.) in PBS (ionic composition in millimoles L^{-1}: Na_2HPO_4 100; NaH_2PO_4 100; NaCl 140; pH 7.4). The stained cells were examined with a standard epi-illumination fluorescence microscope (Axiophot, Zeiss, Germany). Cells stained with propidium iodide represented dead cells, while cells stained with fluorescein diacetate represented live cells. A blinded observer counted the number of dead and alive cells in five microscopic fields (under 40× magnification) reaching approximately 300–450 cells for each coverslip and the mean was regarded as the representative value for the coverslip. The percentage of surviving cells was determined in three or four coverslips for each experimental condition and normalized to controls examined in parallel. The average per cent survival, relative to control, from at least three separate experiments for each condition is expressed in the text and figures as the mean ± s.e.mean. Statistical significance was determined by Student's *t*-test.

GSH and GSSG measurement

Bovine chromaffin cells ($2-4 \times 10^6$ cells/flask) were detached by mechanical procedures. Using plastic pipettes, the cells with accompanying medium were transferred to centrifuge tubes kept on ice and centrifuged ($1000 \times g$; 3 min; 4°C). The cells were resuspended in isotonic PBS to completely washout the culture medium and centrifuged again. Once the supernatant was discarded, the pellet was resuspended (10^6 cells/100 μl in a solution of 5-sulphosalicylic acid (SSA; 3.33%) containing 0.25 mM ethylenediamine tetraacetic acid (EDTA) to prevent oxidation of GSH and to inhibit GSH-utilizing enzymes. Tubes were frozen and thawed three times to break the cells and release glutathione. The lysate was centrifuged ($10{,}000 \times g$; 5 min; 4°C) and the supernatants transferred to eppendorf tubes kept in dry ice until assayed for glutathione content. Glutathione measurements were performed as previously described (Griffith, 1980). GSH reacts non-enzymatically with 5,5′-dithiobis-(2-nitrobenzoic acid) (DNTB) to generate GSSG and the highly coloured 5-thio-2-nitrobenzoic acid (TNB; peak absorbance at 420 nm); the formed GSSG is back reduced to GSH by glutathione reductase coupled to NADPH oxidation. In this cycling assay, concentrations of the reactants are chosen so that the rate of colour formation is linear with time, being the slope of the line Δ absorbance/Δ time, min ($\Delta A/\Delta t$ (min)) directly proportional to total glutathione (GSH + GSSG; GSt) concentration. This allows computer-assisted on-line construction of standard curves relating GSt concentrations to the slopes of their assays. Since this relationship is linear, it is possible to measure the concentration of GSt in identically treated test sample by interpolation. GSSG was similarly determined except for: (1) GSH was masked by derivatization with 2-vinyl pyridine and, (2) the volume of the sample and the concentration of the enzyme (see below) used are higher because the smaller concentration of GSSG than that of GSH.

Standards and tissue homogenates were assayed by triplicate. Assay mixtures contained: NADPH (0.21 mM); DNTB (0.6 mM) and ethylenediamine tetraacetic acid (EDTA) (6.3 mM) in a final volume of 1 ml of phosphate buffer (0.125 M; pH 7.5). Reaction was started by adding the sample (usually 5–10 μl of supernatant for GSt and up to 25 μl for GSSG and GSH; blanks contained a volume identical to the sample of 3.33% SSA) and glutathione reductase (1 to 2 and 5 units for GSt and GSSG assays respectively).

It should be noted that other cellular components containing a sulphhydryl group might react with DNTB, the colour generating reagent in the assay mixture. However, the enzymatic cycling mediated by glutathione reductase provides the specificity to the assay to measure glutathione and prevents the interference by other compounds having the sulphhydryl group. Therefore, the levels of glutathione

measured in chromaffin cells correspond to genuine GSt and GSSG.

Reagents

Tetrodotoxin (TTX), veratridine, xanthine, xanthine oxidase and vitamin E were obtained from Sigma (St. Louis). GSH from Boehringer-Mannheim (Indianapolis, IN, U.S.A.) and MnTBAP from Calbiochem (Schwalbach; Germany). All other reagents were obtained from commercial sources and were of the maximal available purity.

Results

Veratridine induces cytochrome c *release*

We have previously shown that veratridine induced a Ca^{2+}-dependent increase in caspase activity in chromaffin cultures (Jordan *et al*., 2000). Multiple apoptotical pathways release cyt *c* from the mitochondrial intermembrane space and result in the activation of downstream caspases. We were interested to study whether veratridine-induced caspase activation was mediated by cyt *c* release from the mitochondria and if, in this case, O_2^- anions might trigger this release and consequent caspase activation. To address this question we exposed chromaffin cell cultures to 30 μM veratridine for 1 h, and 6 h later we analysed the levels of cyt *c* in cytosolic extracts. Veratridine (30 μM; 1 h) induced cyt *c* release that is TTX-sensitive and Ca^{2+}-dependent (data not shown). In addition, veratridine failed to produce cyt *c* release in presence of the PTP blocker CsA (10 μM). In addition, the superoxide dismutase mimetic MnTBAP (10 nM) (Faulkner *et al*., 1994), catalase (100 IU ml^{-1}) or pretreatment with vitamin E (50 μM), for 1 h, blocked cyt *c* release (Figure 1). Furthermore, paraquat (1 mM, 24 h), a compound that generates O_2^- and that is able to produce apoptotic death in chromaffin cells (Jordan *et al*., 2000), also induced cyt *c* release from mitochondria, being this effect blocked by MnTBAP (10 nM) (Figure 1).

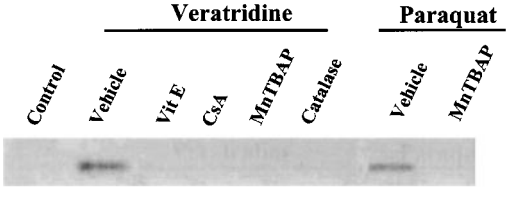

Figure 1 Veratridine induces release of cytochrome *c* from the mitochondria. Cytochrome *c* content in cytoplasmic extracts was measured by Western blot analysis as indicated in Methods. Cytosolic extracts were obtained from untreated (Control) or from chromaffin cells 6 h after veratridine (30 μM; 1 h) exposure (vehicle). For pharmacological studies cultures were pre-treated for 1 h with either vitamin E (50 μM, Vit E), CsA (10 μM, CsA), MnTBAP (10 nM) or catalase (100 IU ml^{-1}) before veratridine treatment. Paraquat (1 mM, Vehicle) was added, in absence of veratridine, to the cellular medium and kept during 6 h in presence or absence of MnTBAP (10 nM). Similar results were found in three separate experiments.

Veratridine-induced caspase activation is mediated by free radicals

One consequence of cyt *c* release is activation of proteases of the caspase family. Consistent with our previous work (Jordan *et al*., 2000), veratridine (30 μM; 1 h) induced caspase activation 6 h after treatment. Herein, we explored whether protease activation following veratridine treatment could be mediated by ROS, by studying the effect of vitamin E on enzymatic activation. As can be observed in Figure 2, 1 h pretreatment with vitamin E protected chromaffin cells against an increase in caspase activity following veratridine. A similar effect was observed in the presence of the PTP blocker CsA (10 μM, 1 h, Figure 2). On the other hand, paraquat (1 mM, 6 h), a compound that generates O_2^-, was also able to induce, by itself, caspase-like activation (data not shown).

Induction of the PTP in isolated mitochondria

In several cell types, mitochondrial PTP opening is required for cyt *c* release (Petit *et al*., 1998; Kantrow & Piantadosi, 1997). In the next set of experiments we aimed to explore whether O_2^- anions might act as inducers of the PTP in chromaffin cells. Induction of PTP opening in isolated chromaffin cell mitochondria was monitored by following absorbance decrease associated with mitochondrial swelling (Bernardi *et al*., 1992). Superoxide induced PTP opening in a dose-dependent manner (Figure 3A). Exposure of mitochondria up to 1 mU XO/X, as superoxide generating system, had no effect, but exposure to 3, 5, 15 and 25 mU XO/X induced a rapid loss of absorbance indicating mitochondrial swelling (Figure 3A). Total decrease in absorbance following exposure to 15 mU XO/X was $23.5 \pm 4.6\%$ ($n=6$). The highest dose of XO/X tested (25 mU, trace 25) did not always produce maximal mitochondrial swelling, suggesting a U-shaped dependence on superoxide as it has been described for calcium (Kristal & Dubinsky, 1997). Superoxide anions-mediated mitochondrial swelling was blocked by compounds known to prevent PTP opening. So, CsA (10 μM) showed inhibitory effects against O_2^--mediated mitochondrial swelling (Figure 3B, trace CsA). However, ruthenium red, a Ca^{2+} uniporter blocker, did not affect O_2^--induced PTP opening

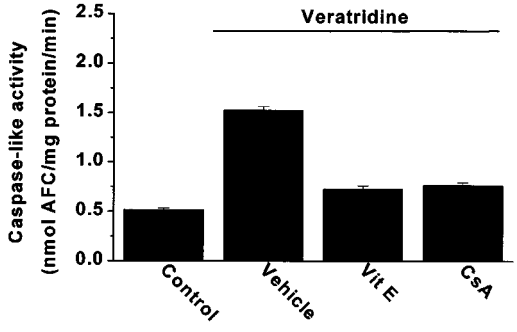

Figure 2 Vitamin E blocks veratridine-induced caspase-like activity. Effect of vitamin E addition on veratridine (30 μM, 1 h)-induced caspase-like activity measured 6 h after veratridine exposure. Vitamin E (50 μM or CsA (10 μM) were added 1 h before and maintained during and after veratridine exposure. Data represent mean $\pm$ s.e.m. of three experiments done by duplicate.

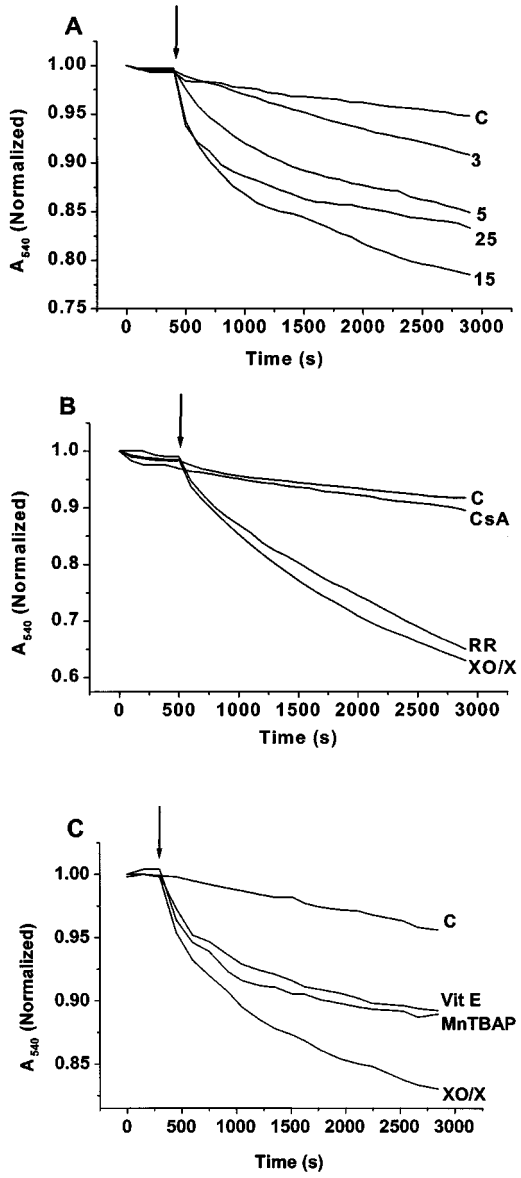

Figure 3 Superoxide anions open PTP. (A) Superoxide-mediated loss in absorbance in isolated chromaffin mitochondria. Different amounts of XO/X system, in a final volume of 25 μl, were added at 5 min as noted by the arrow. Trace C Control, no XO/X added, 25 μl of buffer were added to control for dilution effects; trace 3 (3 mU XO); trace 5 (5 mU XO); trace 15 (15 mU XO); trace 25 (25 mU XO). (B) Effect of cyclosporin A and ruthenium red on O_2^--mediated PTP induction. Mitochondria were incubated during 15 min in absence of drugs (trace C) or in presence of cyclosporin A (10 μM, trace CsA) or ruthenium red (5 μM, trace RR) and the effect of 15 mU XO/X on PTP (trace XO/X) was measured. (C) Effect of vit E (50 μM; trace Vit E), MnTBAP (10 nM, trace MnTBAP) and SOD (450 IU ml^{-1}, Trace SOD) on XO/X (15 mU XO)-mediated PTP opening (trace XO/X). Trace C represents no added drug. Data represent mean values obtained from one experiment performed by triplicate. Similar data were found in at least five different experiments.

suggesting that O_2^--mediated mitochondrial swelling did not require Ca^{2+} influx into mitochondria (Figure 3B, trace RR).

To determine if the observed effects of vitamin E on cyt *c* translocation were also dependent on its ability to block O_2^- anions-induced PTP opening, mitochondria were preincubated for 4 h with vitamin E (50 μM) prior to XO/X exposure. Under these conditions, vitamin E blocked a large portion of the expected PTP opening induced by O_2^- anions (Figure 3C, trace Vit E). The SOD mimetic MnTBAP (10 nM) partially prevented XO/X-induced swelling (Figure 3C; trace MnTBAP) while SOD (100 IU ml^{-1}) showed a smaller effect than MnTBAP on O_2^- anions-induced PTP opening (data not shown) probably due to poor accessibility of the enzyme to mitochondrial domains where O_2^- was produced. Moreover, deferoxamine, an iron chelator (10 mM), did not affect X/XO-induced mitochondrial swelling (data not shown).

Exposure of isolated bovine chromaffin mitochondria to increased concentrations of Ca^{2+} ranging from 25–75 μM induced PTP opening and mitochondrial swelling. This Ca^{2+}-mediated effect was prevented by the Ca^{2+} uniporter blocker ruthenium red (5 μM) and the PTP blocker cyclosporin A (10 μM). However, the non-specific antioxidant vitamin E (50 μM) did not affect Ca^{2+}-mediated mitochondrial swelling, suggesting that free radicals do not contribute to this Ca^{2+}-mediated effect (data not shown).

Vitamin E scavenges veratridine-generated O_2^-

We have previously shown that veratridine induced a dose-dependent increase in O_2^- anion production in chromaffin cells and how this free radical generation required Ca^{2+} influx (Jordan *et al*., 2000). In the present work, we were interested on the role of O_2^- anions as a second messenger in veratridine-induced death. In the next set of experiments, we studied vitamin E scavenger ability on veratridine-induced O_2^- production in chromaffin cells. Cultures were treated with vitamin E (50 μM; 1 h; 37°C) at a concentration effective to prevent veratridine-induced chromaffin cell death (Jordan *et al*., 2000) and O_2^- production was monitored. Bovine chromaffin cells in culture had a basal O_2^- production rate of 6.25 ± 0.61 AFU min^{-1} ($n = 59$; Figure 4). Veratridine (30 μM) caused an increase in the rate of O_2^- production reaching a value of 19.32 ± 2.66 AFU min^{-1} ($n = 102$; Figure

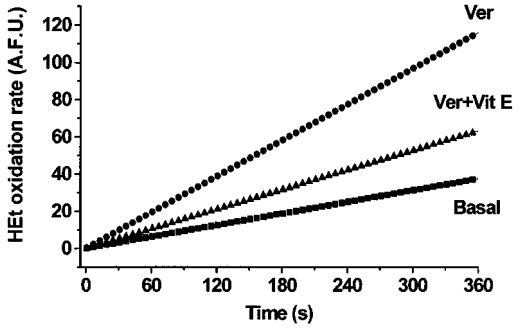

Figure 4 Vitamin E prevents veratridine-induced superoxide anion production. Ethidium fluorescence production following veratridine (30 μM) was determined as indicated in Methods. The slopes of the lines fitting the fluorescence intensity changes (an index of the rate of superoxide production were individually calculated and averaged. The mean slope values were used to generate a theoretical line using the following equation: $y = ax$. The figure represents the generated lines for superoxide production in untreated cells and in the presence of veratridine alone or in presence of vitamin E (50 μM).

4). This O_2^- overproduction was decreased by pretreatment of the cells with 50 μM vitamin E (10.52 ± 1.59 AFU min^{-1}; $n=63$) (Figure 4). These data demonstrate that vitamin E can penetrate the cells and remove O_2^- effectively in this model.

Glutathione levels

Glutathione, a ubiquitous tripeptide thiol, is an endogenous intra- and extracellular protective antioxidant against oxidative stress and plays a key role in the control of apoptotic death. In the next set of experiments we explored whether glutathione levels in chromaffin cells could be affected by veratridine-treatment. Total glutathione level in chromaffin cells amounted to 7.41 ± 1.2 nmoles/5×10^6 cells ($n=6$) most of it (about 85%) as GSH and about 15% as GSSG (Figure 5). Under our experimental conditions, veratridine (30 μM; 1 h) markedly decreased, 24 h later, total glutathione levels to 2.49 ± 0.15 nmoles/5×10^6 cells ($n=7$), half as GSH and half as GSSG (Figure 5). This effect might be due to veratridine-induced ROS generation (Jordan *et al.*, 2000). In agreement with this possibility, treatment of chromaffin cells with H_2O_2 (100 μM; 24 h) decreased total glutathione levels to 2.12 ± 0.32 nmoles/5×1^6 cells ($n=3$) increasing the contribution of GSSG to 80% of this value, amounting GSH to only 20% of total glutathione levels (Figure 5).

In addition, to evaluate the role of a decrease in GSH level on veratridine-induced cell death, we depleted chromaffin cells of GSH using buthionine sulphoximine (BSO). Consistently with the antioxidant cytoprotective role for GSH in chromaffin cells, pre-treatment for 12 h with 100 mM BSO potentiated veratridine-induced death to $42.3 \pm 5.4\%$ ($P<0.01$; $n=4$) (Figure 6). Moreover, in agreement with our previous report (Jordan *et al.*, 2000), N-acetyl cysteine (100 μM), provided protection to chromaffin cultures against veratridine-induced death (Figure 6).

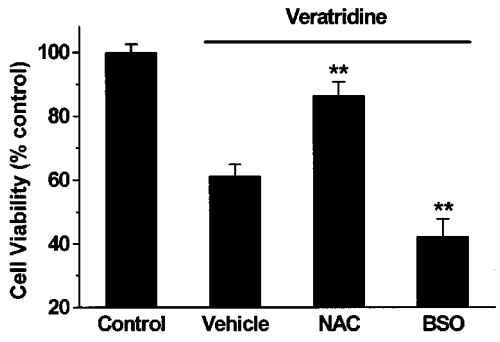

Figure 6 Effect of different agents on chromaffin cell survival 24 h after exposure to veratridine. N-acetyl cysteine (NAC; 100 μM) and BSO (100 μM) were present 12 h before veratridine and maintained during the experiment. Chromaffin cells were treated with veratridine (30 μM; 1 h) and cell viability measured 24 h later as indicated in Methods. Data represent mean $\pm$ s.e.m. of the percentage of living cells after each treatment related to control untreated cells. At least nine coverslips obtained from three different cultures were used for each condition. $^{**}P<0.01$ as compared with veratridine-treated cells in absence of drug.

Discussion

Previous studies from our laboratory have suggested that acute treatment with 30 μM veratridine results in an increase in the rate of O_2^- production, in a Ca^{2+}-dependent manner in bovine chromaffin cells (Jordan *et al.*, 2000). The main objective of the present study was to analyse the contribution of O_2^- to the apoptotic signalling pathway triggered by veratridine in bovine chromaffin cells. Here, we present data that indicate that the increased death produced by veratridine in chromaffin cells is mediated by O_2^- anions overproduction that would produce mitochondrial PTP opening, cyt *c* release and caspase activation.

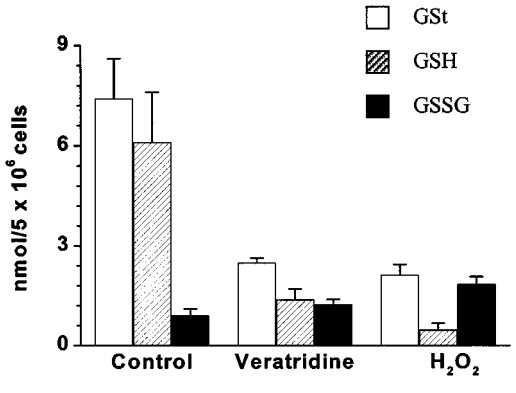

Figure 5 Veratridine and H_2O_2 decreases glutathione levels in chromaffin cells. Cells were treated with veratridine (30 μM; 1 h) or H_2O_2 (100 μM; 24 h) and total glutathione (GSt), reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined 24 h later as indicated in Methods. Data represent mean $\pm$ s.e.m. of 4 to 7 determinations from at least three different cultures.

The results support the hypothesis that veratridine-induced cellular death is associated with disturbances of cellular free radical homeostasis. Thus, chromaffin cells exposed to 30 μM veratridine, present a temporal pattern of apoptotic events following O_2^- elevation, where mitochondrial PTP opening and cyt *c* release preceded caspase activation. We used isolated bovine chromaffin mitochondria to investigate the direct effects of O_2^- anions on mitochondrial PTP, by using the enzymatic reaction of xanthine with xanthine oxidase as an '*in vitro*' O_2^--generating system. Bovine chromaffin cell mitochondria responded to O_2^- anions by opening PTP in a similar way as they do in response to Ca^{2+} ions (Gunter & Gunter, 1994). Moreover, Ca^{2+}-induced PTP opening is not affected by vitamin E, suggesting that O_2^- anions and Ca^{2+} might act through different ways to open PTP. Nevertheless, a common point in both pathways is the blocking ability of cyclosporin A. However, O_2^- effects on chromaffin cell mitochondrial PTP seem to be Ca^{2+}-independent. Moreover, the presence of ruthenium red, a blocker of mitochondrial Ca^{2+} uniporter did not modify O_2^--induced PTP opening, while the pore blocker, cyclosporin A, markedly inhibited the opening process. Pretreatment with vitamin E for 4 h produced a blocking effect in O_2^--induced pore opening. It is important to note that vitamin E effects require a pretreatment to prevent O_2^--induced mitochondrial PTP opening. This time would be necessary to allow the antioxidant to be internalized in the mitochondrial intermembrane space to protect against oxidative O_2^- damage.

Under some conditions, PTP activation appears to be irreversible and has profound consequences for cell function,

being associated with release of molecules into the cytoplasm, like apoptosis inducing factor and cyt *c*, that modulate the execution phase of apoptosis, likely through activation of caspases (Bernardi *et al*., 1999; Kroemer *et al*., 1997). The present results strongly suggest that O_2^--generated intracellularly by veratridine is able to trigger a mitochondria-dependent apoptotic pathway. Veratridine induces release of cyt *c* from chromaffin cell mitochondria and, 6 h after treatment, cyt *c* can be found in chromaffin cell cytosol. The involvement of PTP in this process seems clear since cyclosporin A, a mitochondrial PTP inhibitor, was able to block veratridine-mediated cyt *c* release. Furthermore, ROS might contribute to cyt *c* release because vitamin E, MnTBAP and catalase prevented veratridine-induced cyt *c* release. In addition, paraquat, an O_2^--generating compound, that produces apoptotic death in chromaffin cells (Jordan *et al*., 2000), also induced cyt *c* release. Once released, cyt *c* may contribute to apoptotic death by regulating caspase activation. In chromaffin cells this seems to be the case because either vitamin E or cyclosporin A, that block cyt *c* release, prevented veratridine-induced caspase-like enzymatic activation.

Veratridine treatment results in a deregulation of $[Na^+]_i$ and $[Ca^{2+}]_i$, and as a consequence of the latter, collapse of transmembrane potential difference and O_2^- anions overproduction that can feed back into the mitochondria to amplify the process as it has been described in models where the O_2^- scavenger ability of the cell is depleted, resulting in apoptosis induction (Kokoszka *et al*., 2001). Moreover, H_2O_2 produced during O_2^- processing might play a role in veratridine-induced toxicity because catalase blocks cyt *c* release and also it has been described that catalase (100 IU ml^{-1}) partially prevents veratridine-induced chromaffin cell death (Jordan *et al*., 2000). In addition, O_2^- might react avidly with nitric oxide (NO) to produce the powerful oxidizing agent, peroxynitrite ($ONOO^-$) that affects mitochondrial functions mostly in an irreversible manner. This mechanism might be activated in our model because the nitric oxide synthase inhibitor L-nitro arginine partially prevents veratridine-induced chromaffin cell death (Jordan *et al*., unpublished results).

Tripeptide reduced glutathione (GSH) acts as a free radical scavenger and is important in recycling other antioxidants (Sies, 1993). Veratridine markedly decreased chromaffin GSH levels. Recent findings have suggested that GSH is important in immune modulation, remodeling of the extracellular matrix, apoptosis and mitochondrial respiration (Fernandez-Checa *et al*., 1998; Si *et al*., 1998; Schnelldorfer *et al*., 2000). It is important to note that, regardless of the oxidant(s) responsible for veratridine-induced death, the molecular targets of this oxidant(s) are likely to include intracellular thiols such as GSH, since the alkaloid markedly reduced both total glutathione and GSH in chromaffin cells.

In summary, O_2^- radicals generated by veratridine open mitochondrial PTP, induce cyt *c* release from mitochondria and activate caspases in chromaffin cells. These actions of O_2^- anions are prevented by non-specific antioxidants like vitamin E. It would be of interest to explore if other free radical species, different from O_2^-, might contribute to veratridine-induced death in bovine chromaffin cells.

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