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ORIGINAL ARTICLE

Role of intracellular calcium on hydrogen peroxide-induced apoptosis in rat pancreatic acinar AR42J cells

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Summary
The authors investigated whether cytosolic free calcium concentration ($[Ca^{2+}]c$) plays a role in hydrogen peroxide-induced pancreatic acinar AR42J cells apoptosis. We analysed mitochondrial depolarization, $[Ca^{2+}]c$ determination and caspase-3 activity by fluorimetric methods, and cytochrome c release by subcellular fractionation and western blotting. The data shown that hydrogen peroxide, which causes a sustained $[Ca^{2+}]c$ increase, induces mitochondrial depolarization and cytochrome c release, and activation of caspase-3. Dimethyl-BAPTA loading did not affect hydrogen peroxide-evoked mitochondrial apoptosis, suggesting that these responses are independent of increases in $[Ca^{2+}]c$. Treatment with thapsigargin, to induce extensive calcium store depletion and subsequent increases in $[Ca^{2+}]c$, also stimulates mitochondrial depolarization cytochrome c release, and caspase-3 activation. Similar results were observed in AR42J cells loaded with dimethyl-BAPTA, suggesting that activation of apoptosis by thapsigargin does not require rises in $[Ca^{2+}]c$. However, the blockade of mitochondrial calcium entry by pretreating with Ru360 showed protection against hydrogen peroxide- and thapsigargin-induced mitochondrial apoptosis. These results indicate that the apoptosis evoked by hydrogen peroxide and thapsigargin is mediated by mitochondrial calcium uptake.

Key words: programed cell death – caspase-3 – cytochrome c – mitochondrion – thapsingargin – dimethyl-BAPTA – Ru360

INTRODUCTION

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The AR42J cell line is the only cell line currently available that maintains many characteristics of normal pancreatic acinar cells, such as the synthesis and secretion of digestive enzymes (Scemama et al. 1987). In fact, AR42J cell receptor expression and signal transduction mechanisms parallel those of pancreatic acinar cells (Scemama et al. 1987). Thus, this cell line has been widely used as an "*in vitro*" model to study cellular secretion, growth, proliferation, and apoptosis of the exocrine pancreas (Scemama et al. 1987, Christophe 1994, Sata et al. 1997, Song et al. 2003, Yu et al. 2005).

One of the earliest and most consistently observed features of apoptosis or programmed cell death is the activation of a series of cytosolic cysteine proteases, called caspases, which cleave multiple protein substrates *en masse*, leading to the loss of cellular structure and function, and ultimately resulting in cell death (Stennicke and Salvesen 1997). The caspase family consists of at least 14 enzymes in mammalian cells. In particular, caspases-3, -8 and -9 play a relevant role in apoptosis: caspase-9 in the mitochondrial pathway, caspase-8 in the Fas/CD95 pathway, and caspase-3, more downstream, is an executioner caspase activated by multiple pathways (Shi 2002).

Traditionally, two general pathways to apoptosis have been described. One pathway is the so-called extrinsic pathway initiated by the binding of an extracellular death ligand, such as FasL, to its cell-surface death receptor, such as Fas (Ashkenazi and Dixit 1998). The second pathway is the intrinsic pathway, which is mediated by mitochondrial alterations. In response to apoptotic stimuli, several proteins are released from the intermembrane space of mitochondria into the cytoplasm (Green and Reed 1998). These well-characterized proteins include cytochrome c, which mediates the activation of caspase-9 (Li et al. 1997), triggering in turn a cascade of caspase activation, including caspase-3, which promotes cellular apoptosis.

On the other hand, whereas ion calcium is a key regulator of cell survival, the sustained elevation of cytosolic free calcium concentration ($[Ca^{2+}]c$) plays a role in cell death (Demaurex and Distelhorst 2003). The pro-apoptotic effects of calcium are mediated by a diverse range of calcium-sensitive factors that are compartmentalized in various intracellular organelles including endoplasmic reticulum and mitochondria (Hajnoczky et al. 2000). Excessive calcium load to the mitochondria may induce apoptosis by stimulating the release of apoptosis promoting factors from the mitochondrial intermembrane space to the cytoplasm and by impairing mitochondrial function (Wang 2001).

Apoptosis can also be stimulated by reactive oxygen species (ROS) in several cell types (Suzuki et al. 1997, Tan et al. 1998, Brookes et al. 2004). Recently, it has been reported that ROS can mediate the apoptosis induced by growth factors, such as transforming growth factor-β (TGF-β) in human lens epithelial cells (Yao et al. 2007), classical agonists, such as thrombin in human platelets (Lopez et al. 2007) or progesterone in human spermatozoa (Bejarano et al. 2008).

Although ROS and calcium have been separately reported to be important mediators of apoptosis, little correlation between these two mediators has been reported. Here, we focused on the role of calcium in hydrogen peroxide (H_2O_2)-induced apoptosis in the pancreatic acinar cell line AR42J and investigated the effects of the calcium chelator 1,2-bis-(o-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester (BAPTA-AM) and of the mitochondrial calcium uptake blocker Ru360 on H_2O_2-evoked apoptosis, analyzing mitochondrial depolarization, caspase-3 activation and cytochrome c release.

MATERIALS AND METHODS

Materials

AR42J cell line (ECACC N° 93100618) derived from exocrine pancreatic rat tumour was purchased from The European Collection of Cell Cultures (ECACC) (Dorset, U.K.). Fetal bovine serum (FBS) from HyClone (Spain). Glutamine, RPMI 1640 medium and penicillin/streptomycin were obtained from BioWhittaker (Madrid, Spain). Thapsigargin (Tg), dexamethasone, Sigma TOX-7, N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methyl-coumarin (AC-DEVD-AMC), dithiothreitol (DTT), and dimethyl BAPTA were from Sigma (Madrid, Spain). Ru360 was from Calbiochem (Nottingham, U.K.). Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) from GIBCO (Barcelona, Spain). An ApoAlert® cell fractionation kit was purchased from BD Biosciences-Clontech (Madrid, Spain). Fura-2 acetoxylmethyl ester (fura-2 AM) and tetramethylrhodamine methyl ester perchlorate (TMRM) were obtained from Molecular Probes Europe (Barcelona, Spain). All other reagents were of analytical grade.

Cell culture

AR42J cells derive initially from a transplantable tumour of a rat exocrine pancreas. This line is tumourigenic in nude mice, and shows a significant secretion of amylase and other exocrine enzymes. AR42J cells (passages 6–12) were cultured in RPMI 1640 supplemented with 2 mM glutamine, 10% FBS and antibiotics (0.1 mg/ml streptomycin, 100 IU penicillin) at 37 °C under a humidified condition of 95% air and 5% CO_2. Cells were routinely plated at a density of ~105 cells/ml into fresh flasks. Dexamethasone treatment has been found to convert these cells into exocrine cells (Eum et al. 2003). AR42J is as yet the only cell line that exhibits many characteristics of normal pancreatic acinar cells

such as synthesis and secretion of digestive enzymes. Thus, this line has been used to study secretion, growth, proliferation, and oxidant-induced apoptosis of exocrine pancreatic cells (Yu et al. 2005). After incubation with 100 nM dexamethasone for 48 h, the cells were washed with PBS and incubated in fresh medium. The experiments were started by the addition of H_2O_2 at doses ranging 1 μM, 10 μM and 100 μM. After 6–48 h, cells were detached with 0.2% trypsin-EDTA.

Cell viability assay

Cell damage was determined by the release of lactate dehydrogenase (LDH) into the incubation medium under different conditions. A commercially prepared kit (Sigma TOX-7) was employed in this assay, and determinations were performed according to manufacturer's instructions. LDH activity in the supernatant was calculated as percent of the total content of LDH at the beginning of the incubation period. For statistical analysis, data of LDH release under the different treatment were compared to the values obtained in control (non-stimulated) cells. Treatment of cells with 10 μM H_2O_2 did not induce any significant increase in LDH activity in the supernatant after 24 h of incubation compared to that found in control (non-stimulated) cells, indicating that the cell damage was not significantly affected by the treatment with H_2O_2. The calculated percentage of LDH release with respect to that found in the control was 1.82±0.6% (n=4). We only found statistically significant differences at the concentration of 1 mM H_2O_2 or greater, which induced an increase of 8.87±1.68% (n=5) as compared to the control cells (statistically significant).

Caspase activity assay

To determinate caspase-3 activity, stimulated or resting cells were sonicated and cell lysates were incubated with 2 ml of substrate solution (20 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid (HEPES), pH 7.4, 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM DTT and 8.25 μM of caspase-3 substrate) for 2 h at 37 °C as previously described (Rosado et al. 2006). Substrate cleavage was measured with a fluorescence spectrophotometer with an excitation wavelength of 360 nm and emission at 460 nm. The activity of caspase was calculated from the cleavage of the specific fluorogenic substrate (AC-DEVD-AMC). The data were calculated as fluorescence units/mg protein and presented as a percentage relative to the control.

Subcellular fractionation

Subcellular fractionation was performed using a commercial mitochondria isolation kit (ApoAlert® cell fractionation kit, BD Biosciences-Clontech, Madrid, Spain) and following the manufacturer's instructions. Briefly, cells were washed in the ice-cold Wash Buffer, resuspended in the Fractionation Buffer Mix containing protease inhibitor cocktail and 1 mM DTT and then lysed in an ice-cold dounce tissue grinder (25 strokes). Homogenates were centrifuged at 200 g for 5 min at 4 °C. The supernatant was further subjected to centrifugation at 10,000 g for 10 min at 4 °C to yield the mitochondrial pellet (which was resuspended in the Fractionation Buffer Mix) and the mitochondria-free cytosolic fraction (supernatant). The cytosolic and mitochondrial fractions were collected separately and used for Western blotting.

Western blotting

One-dimensional sodium dodecyl sulfate (SDS)-electrophoresis was performed with 15% SDS-PAGE and the separated proteins were electrophoretically transferred for 2 h at 0.8 mA/cm^2, in a semi-dry blotter onto nitrocellulose for subsequent probing. Blots were incubated overnight with 10% (w/v) bovine serum albumin (BSA) in tris-buffered saline with 0.1% Tween 20 [tris buffered saline with Tween 20 (TBST)] to block residual protein binding sites. Blocked membranes were then incubated with the anti-cytochrome c antibody diluted 1:100 in TBST for 1 h. The primary antibody was removed and blots washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with the appropriate horseradish peroxidase-conjugated anti-IgG antibody diluted 1:10,000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were then exposed to photographic films and the optical density was estimated using scanning densitometry.

Cell loading and $[Ca^{2+}]c$ determination

Cells were loaded with the fluorescent ratiometric calcium indicator fura-2 by incubation with fura-2 AM (1–2 μM, for 30 min, at room temperature), as we described previously (Lajas et al. 1998). Once loaded, the cells were washed and used within the next 2–4 hours.

Fluorescence was recorded from 2 ml aliquots

of magnetically stirred cell suspensions at 37 °C using a fluorescence spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Cells were previously resuspended in a Na-HEPES buffer containing (in mM): 140 NaCl, 4.7 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose and pH adjusted to 7.4. In experiments where a calcium-free medium is indicated, calcium was omitted and 1 mM ethylene glycol-bis (2-aminoethylether)-N,N,N',N' -tetraacetic acid (EGTA) was added. Changes in $[Ca^{2+}]c$ were monitored using the Fura-2 340/380 fluorescence ratio and calibrated to the method of Grynkiewicz et al. (1985).

Mitochondrial membrane potential (Ψm) determination

Changes in Ψm were registered using the dye TMRM and a confocal laser scanning fluorescence, as described previously (González et al. 2003). AR42J cells were incubated during 30 min in the presence of 100 nM TMRM at 37°C. At the concentration used in our conditions, TMRM accumulates within mitochondria driven by the membrane potential but autoquenching is negligible. A decrease in TMRM fluorescence reflects depolarization of Ψm, due to diffusion of the dye to the cytosol. No TMRM was added to the medium after the initial loading period. Fluorescence images from cells loaded with TMRM were obtained using a laser scanning confocal microscope with ×60 oil-immersion objectives. TMRM was excited by the 543-nm line of He-Ne laser and emission was collected through a 605/32-nm band-pass filter. Results obtained in TMRM-loaded cells are expressed as the absolute values of fluorescence emission at the excitation wavelength employed. Data were normalized previously to the basal (resting) fluorescence values.

Statistical Analysis

Data are present as mean ± standard error of mean (S.E.M.) and analysis of statistical significance was performed using Student's t-test at the significance level $2\alpha=0.05$.

RESULTS

H_2O_2 induces activation of caspase-3 and mitochondrial membrane depolarization in AR42J cells

To examine the effect of H_2O_2 on caspase-3 activation, pancreatic acinar AR42J cells were treated with increasing concentrations of H_2O_2 (1 μM, 10 μM and 100 μM) for 6–48 hours. As shown in Fig. 1a, treatment of AR42J cells with H_2O_2 for 24 hours induced a concentration-dependent activation of caspase-3. Cell stimulation with H_2O_2 caused a detectable activation of caspase-3 at 1 μM with a 147 ± 46% of the control, representing a 47% increase, and the maximum effect was obtained at a dose of 100 μM (211 ± 82% of the control, statistically significant). In addition, treatment for 24 h with 1 μM thapsigargin, a sarco-endoplasmic reticulum calcium-ATPase (SERCA) inhibitor which depletes the intracellular calcium stores, also increased the caspase-3 activity (Fig. 1a). Activation of caspase-3 by thapsigargin was greater in magnitude than the increases obtained with H_2O_2.

As shown in Fig. 1b, the effect of H_2O_2 on caspase-3 activation was also time-dependent, reaching a maximal caspase activity after 24 h of stimulation (191 ± 47% of the control, statistically significant) at the concentration of 10 μM. After this point, caspase activity decreased.

In a calcium-free medium, treatment of AR42J cells with 10 μM H_2O_2 induced a mitochondrial membrane depolarization as detected by the decrease in TMRM fluorescence (Fig. 2a). In cells loaded with the intracellular calcium chelator dimethyl BAPTA, by incubating the cells for 30 min at 37 °C with 10 μM dimethyl BAPTA-AM, and suspended in calcium-free medium, 10 μM H_2O_2 exerted a similar effect on mitochondrial membrane potential (Fig. 2c), suggesting that increases in $[Ca^{2+}]c$ are not required for H_2O_2-evoked mitochondrial depolarization. Treatment with 1 μM thapsigargin also induced decreases in TMRM fluorescence that were independent of rises in $[Ca^{2+}]c$ (Fig. 2b and 2d).

H_2O_2 induces cytochrome c release in AR42J cells

To determine whether mitochondrial cytochrome c translocates from the mitochondria to the cytosol, mitochondrial and cytosol fractions were collected following exposure to H_2O_2 for 24 hours. Our results shown that treatment with 10 μM H_2O_2 for 24 h is able to induce cytochrome c release in AR42J cells (Fig. 3). Compared with untreated cells, treatment with H_2O_2 stimulated significantly the cytochrome c level in the cytosol (300 ± 97% of the control, statistically significant), with a concomitant decrease in the mitochondrial cytochrome c content (69 ± 2% of the control, statistically significant). Similar results were obtained when the cells were treated with 1 μM thapsigargin for 24 h (Fig. 3) (509 ±136 versus 37 ±8 % of the control of cytochrome c content in the cytosolic and

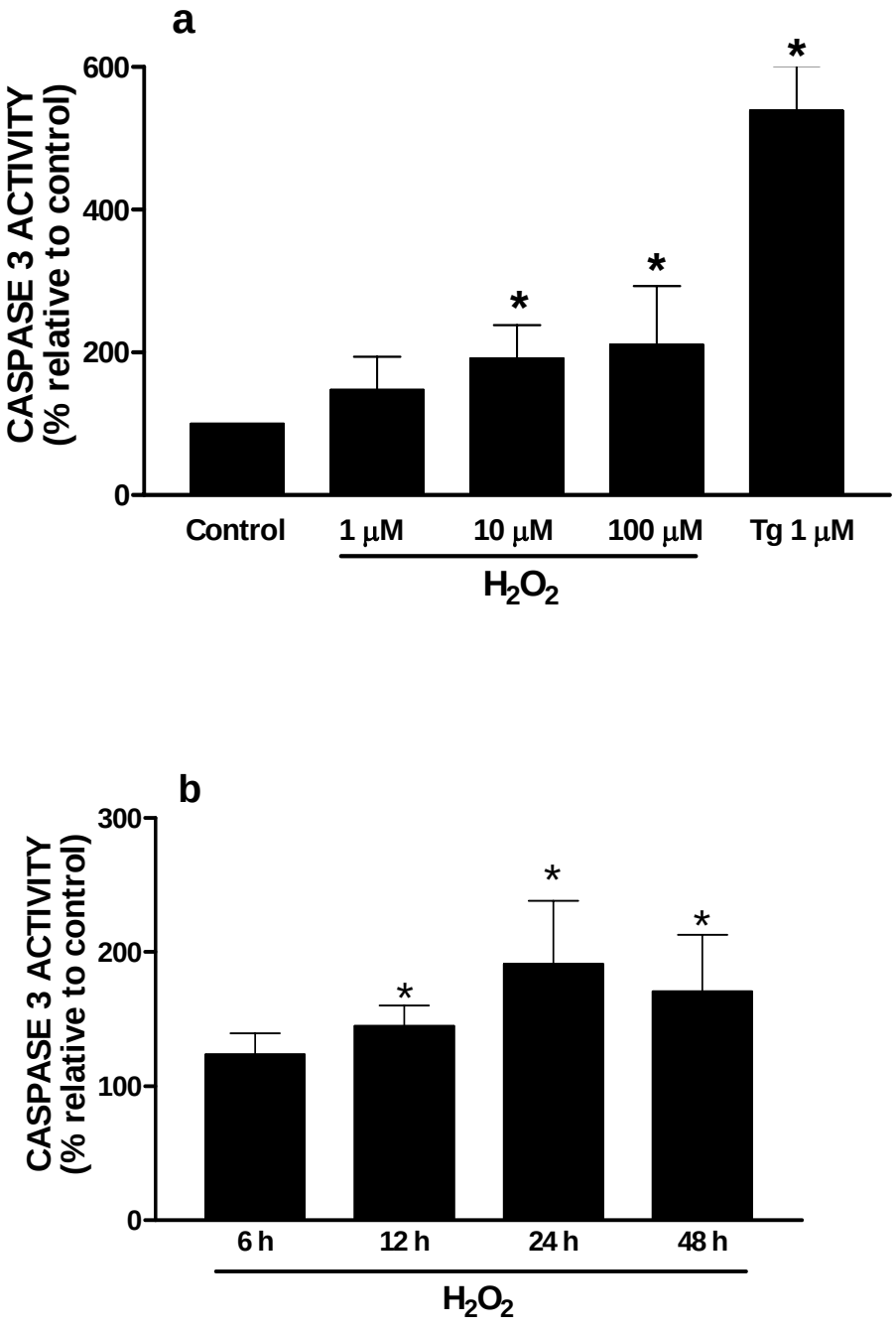

Fig. 1. **Concentration dependence and time course of H_2O_2-induced activation of caspase-3.** AR42J cells were stimulated for 24 h with increasing concentrations of H_2O_2 (1 μM, 10 μM and 100 μM) (a) or for various periods of time (6h, 12 h, 24 h and 48 h) with 10 μM H_2O_2 (b). The effect of 1 μM thapsigargin (Tg) treatment for 24 h on activation of caspase-3 is included in (a). Caspase-3 activity was estimated as described under "Material and Methods". Values are presented as means + S.E.M. of 5–8 separate experiments and expressed as percentage relative to control (untreated samples); * statistically significant as compared with control values.

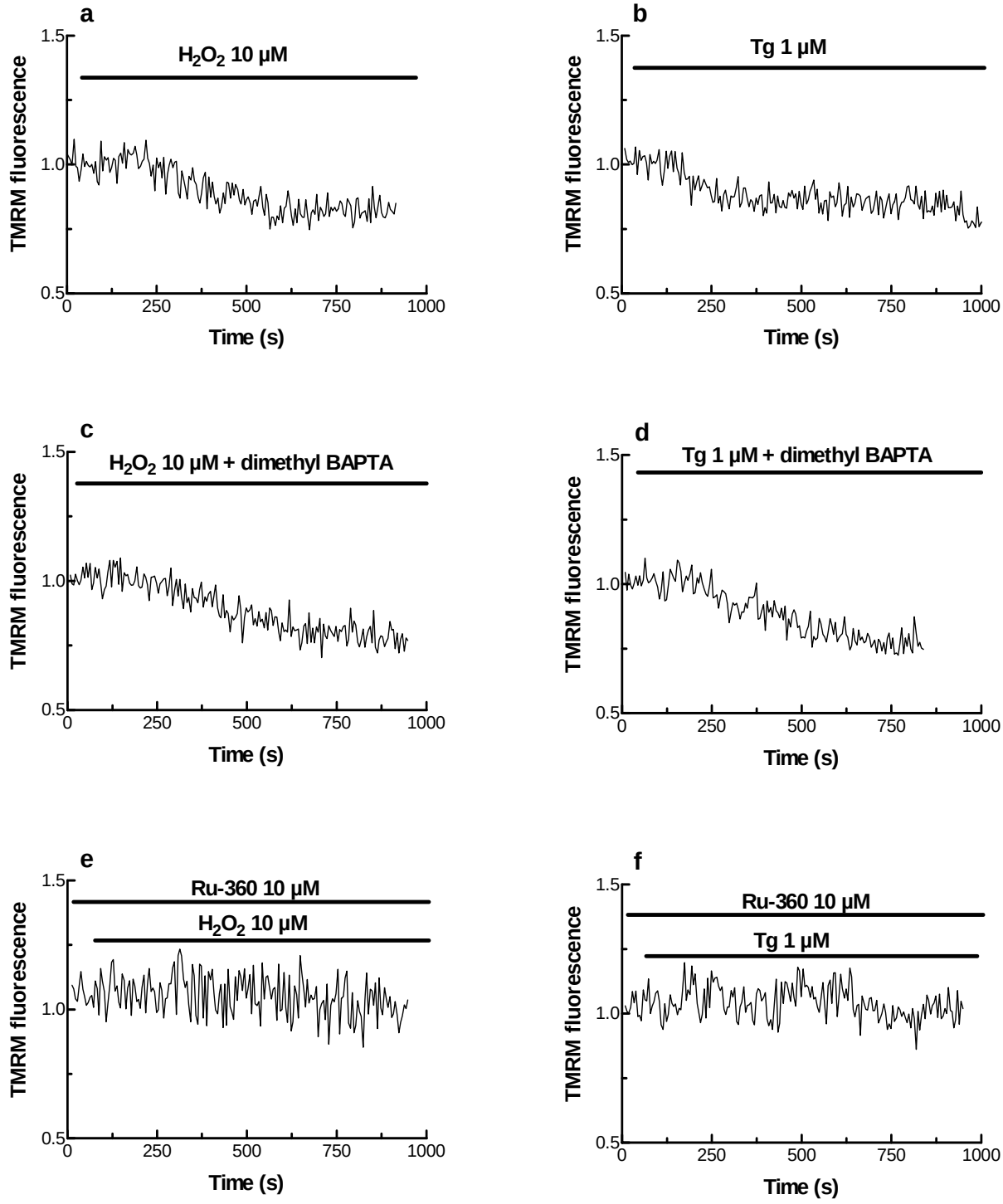

Fig. 2. **H_2O_2 induces mitochondrial membrane depolarization in AR42J cells.** Control (a and b), dimethyl BAPTA (10 µM for 30 min)-loaded (c and d) or Ru360 (10 µM for 30 min)-pretreated (e and f) cells were loaded with TMRM. Cells were then stimulated with 10 µM H_2O_2 (a, c and e) or 1 µM thapsigargin (Tg) (b, d and f) in the presence of 10 µM Ru360 (e and f), in a calcium-free medium (1 mM EGTA was added). Changes in mitochondrial membrane potential were determined as shown under "Material and Methods". Traces are representative of 25–38 cells.

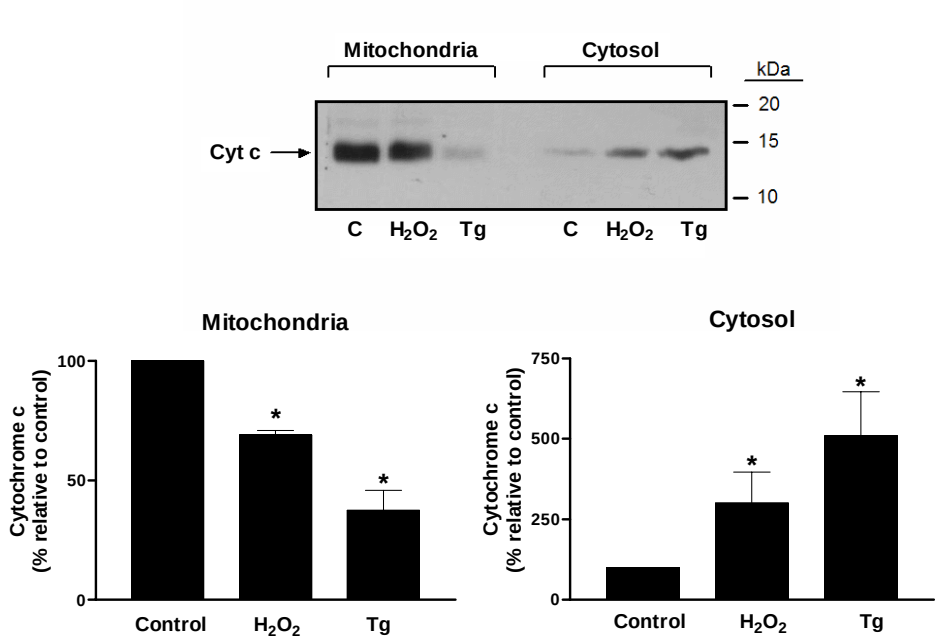

Fig. 3. **H_2O_2 induce cytochrome c release.** AR42J cells were stimulated with H_2O_2 (10 μM) or thapsigargin (Tg) (1 μM) for 24 h. The cytochrome c content in the cytosolic and mitochondrial fractions was determine by Western blotting as described in "Material and Methods". Panels show a blot representative of three others. Histograms represent the change in cytochrome c content and is presented as percentage relative to control (untreated samples). Values are represented as means ± S.E.M. of 3 separate experiments; *statistically significant as compared with control (untreated samples) values.

mitochondrial fractions, respectively, statistically significant), though thapsigargin was also found to be a more potent activator of cytochrome c release than H_2O_2.

Involvement of calcium signal in H_2O_2-induced caspase activation and cytochrome c release

It has been reported that a prolonged elevation in $[Ca^{2+}]c$ and alterations in calcium homeostasis initiate the mitochondrial apoptotic pathway (Hajnoczky et al. 2003) and induce endoplasmic reticulum stress that, in turn, leads to apoptosis (Rao et al. 2004). In the presence of normal extracellular calcium concentration, treatment of AR42J cells with 10 μM H_2O_2 caused a slow and sustained $[Ca^{2+}]c$ increase, which reached a stable $[Ca^{2+}]c$ plateau after 8-10 minutes of stimulation (Fig. 4a). Fig. 4b demonstrates that the increase of $[Ca^{2+}]c$ induced by H_2O_2 was also observed in a calcium-free medium, reflecting the release of calcium from intracellular stores.

To determine the role of rises in $[Ca^{2+}]c$ in caspase-3 activation and cytochrome c release, we performed a series of experiments where AR42J cells were loaded with dimethyl BAPTA-AM (10 μM for 30 min). As expected, buffering of cytosolic calcium with BAPTA prevented H_2O_2-evoked $[Ca^{2+}]c$ elevations (Fig. 4b), but did not modify significantly H_2O_2-induced caspase-3 activation and cytochrome c release from the mitochondria. As shown in Fig. 5, after stimulation with 10 μM H_2O_2 for 24 h, the activity of caspase-3 was 156 ± 34% of the control in dimethyl BAPTA-loaded cells and 198 ± 47% of the control in absence of dimethyl BAPTA. In addition, dimethyl BAPTA loading had no significant effects on cytochrome c release induced by H_2O_2 (Fig. 6). These findings indicate that changes in $[Ca^{2+}]c$ are not required for H_2O_2-induced caspase-3 activation and cytochrome c release.

To further investigate the role of $[Ca^{2+}]c$ in caspase activation and cytochrome c release, we used the pharmacological tool thapsigargin, a specific inhibitor of SERCA, to deplete the intracellular calcium stores. Treatment of AR42J cells, in a calcium-free medium, with 1 μM thapsigargin induced an extensive depletion of the

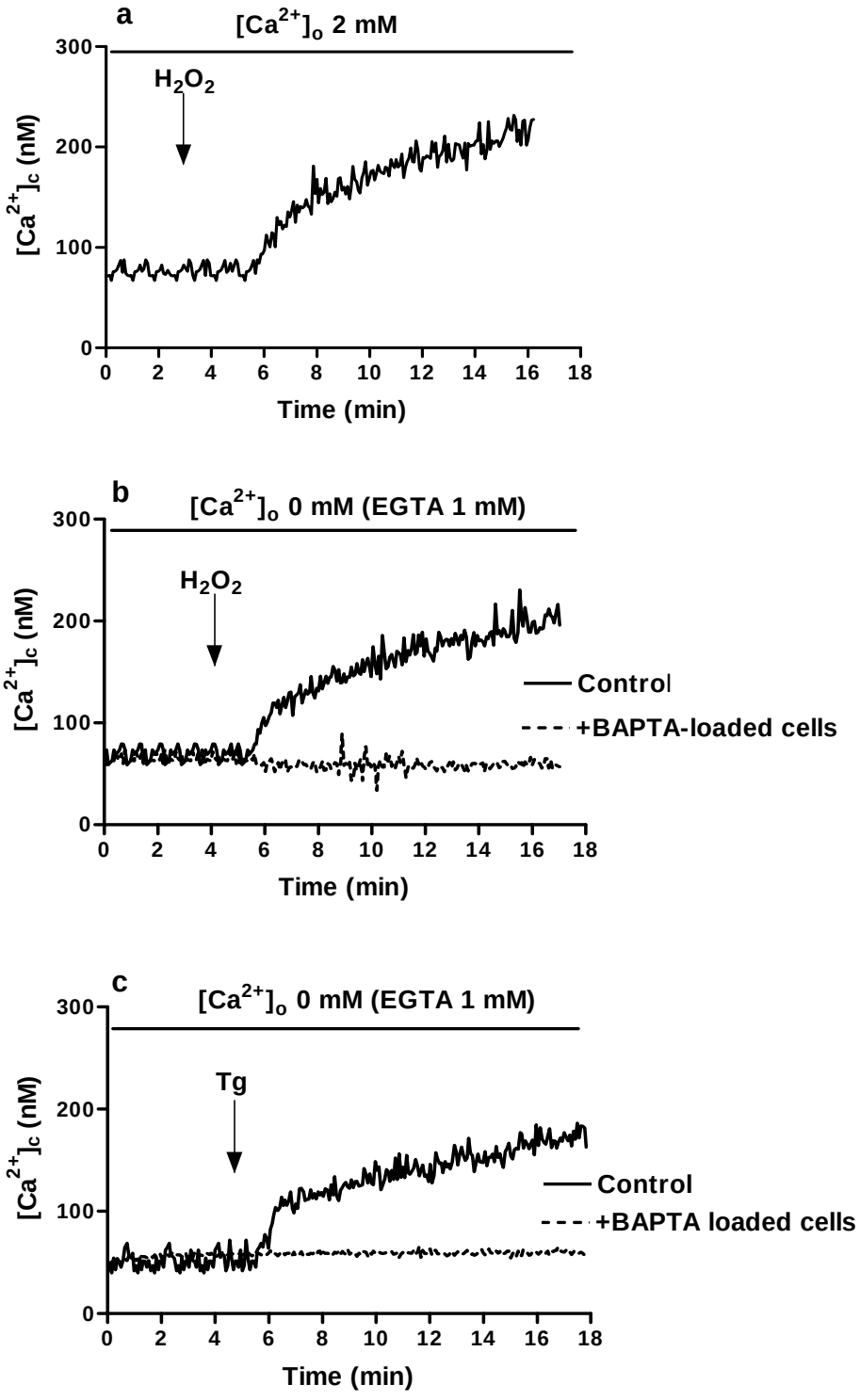

Fig. 4. **Mobilization of calcium in response to H_2O_2 in AR42J cells.** Cells were stimulated with 10 μM H_2O_2 (a and b) or 1 μM thapsigargin (Tg) (c) in calcium-normal (a) or – free (b and c) solution (1 mM EGTA was added) in the presence or absence of 10 μM dimethyl BAPTA. Traces are representative of 5-9 independent experiments.

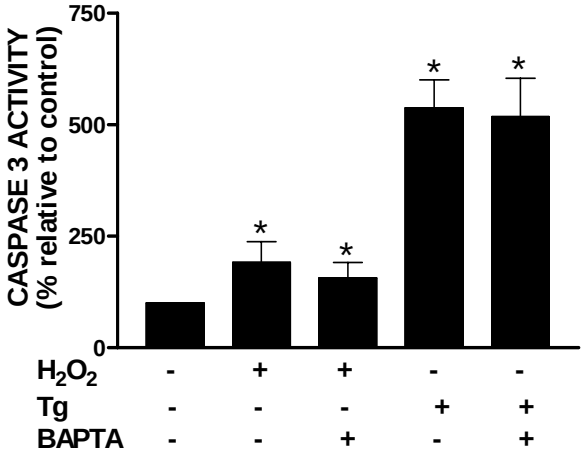

Fig. 5. **Effect of intracellular calcium chelation on H_2O_2-induced caspase-3 activation.** Control or dimethyl BAPTA (10 μM for 30 min)-loaded AR42J cells were stimulated for 24 h with 10 μM H_2O_2 or 1 μM thapsigargin (Tg). Caspase-3 activity was estimated as described under "Material and Methods". Values are presented as means ± S.E.M. of 5–10 separate experiments and expressed as percentage relative to control (untreated samples); *statistically significant as compared with control (untreated samples) values.

intracellular calcium stores and subsequent increase in $[Ca^{2+}]c$, which were prevented by dimethyl BAPTA loading (Fig. 4c). In cells loaded with dimethyl BAPTA, the effect of 1 μM thapsigargin for 24 h on caspase-3 activation was similar to that observed in its respective control (in thapsigargin-stimulated cells, the activity of caspase-3 was 518 ± 86% of the control in dimethyl BAPTA loaded cells, and 538 ± 63% of the control in absence of dimethyl BAPTA; Fig. 5). The results obtained by treatment with thapsigargin in dimethyl BAPTA loaded cells were confirmed using the cytochrome c release assay (Fig. 6). These data exclude the possibility that the thapsigargin-elicited rise in $[Ca^{2+}]c$ results in caspase-3 activation and cytochrome c release from the mitochondria.

Prevention of H_2O_2-induced apoptosis by the blockade of calcium uptake into mitochondria

To determine whether H_2O_2-induced apoptotic signals are mediated by the calcium uptake into mitochondria, we investigated the effect of the blocker of calcium entry into mitochondria Ru360, by preincubation of the cells for 30 min with 10 μM Ru360, on H_2O_2- and thapsigargin-induced caspase-3 activation and cytochrome c release from the mitochondria. As shown in Fig. 7, the blockade of mitochondrial calcium entry decreases significantly H_2O_2-induced caspase-3 activation. In Ru360-treated cells, the caspase-3 activity was ~ 30% of the effect observed with H_2O_2 alone (Ru360-untreated cells) (108 ± 17% of the control versus 191 ± 47% of the control, statistically significant as compared with statistically significant, Fig. 7). Pretreatment with Ru360 was also able to prevent H_2O_2- and thapsigargin-evoked mitochondrial membrane depolarization (Fig. 2e and 2f). Additionally, the release of mitochondrial cytochrome c in Ru360 treated cells was significantly decreased compared with cells stimulated with 10 μM H_2O_2 for 24 h (Fig. 8). Similar results were obtained when apoptosis signals were stimulated with 1 μM thapsigargin for 24 h. In the presence of Ru360, thapsigargin-evoked caspase-3 activation and cytochrome c release was significantly inhibited (Fig. 7 and 8). Taken together, these results indicate that the calcium uptake into mitochondria is required for H_2O_2- and thapsigargin-induced mitochondrial apoptosis.

DISCUSSION

Apoptosis or programmed cell death is a genetically regulated process essential for maintaining tissue homeostasis and for the elimination of undesirable

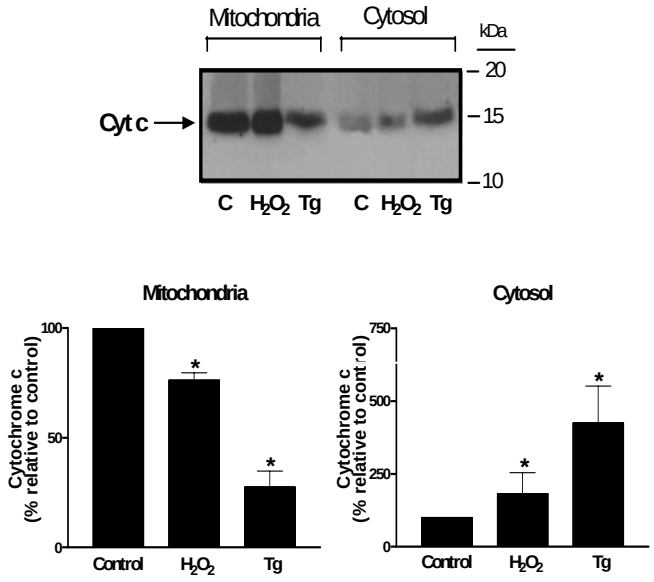

Fig. 6. **Effect of intracellular calcium chelation on H_2O_2-induced cytochrome c release.** Dimethyl BAPTA (10 μM for 30 min)-loaded AR42J cells were incubated for 24 h with H_2O_2 (10 μM) or thapsigargin (Tg) (1 μM). The cytochrome c content in the cytosolic and mitochondrial fractions was determine by Western blotting as described in "Material and Methods". Panels show a blot representative of three others. Histograms represent the change in cytochrome c content and is presented as percentage relative to control (dimethyl BAPTA treated samples). Values are represented as means ± S.E.M. of 3 separate experiments. *statistically significant as compared with control (dimethyl BAPTA treated samples) values.

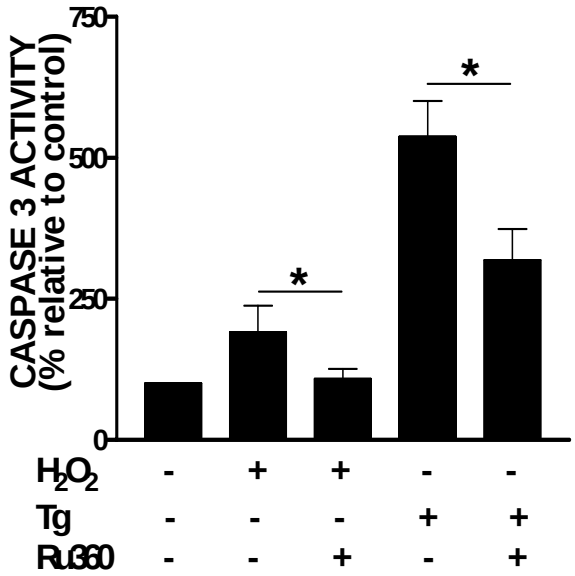

Fig. 7. **Effect of blockade of calcium uptake into mitochondria on H_2O_2-induced caspase-3 activation.** Control or Ru360 (10 μM for 30 min)-pretreated AR42J cells were stimulated for 24 h with 10 μM H_2O_2 or 1 μM thapsigargin (Tg). Caspase-3 activity was estimated a described under "Material and Methods". Values are presented as means ± S.E.M. of 6–8 separate experiments and expressed as percentage relative to control (untreated samples); *statistically significant

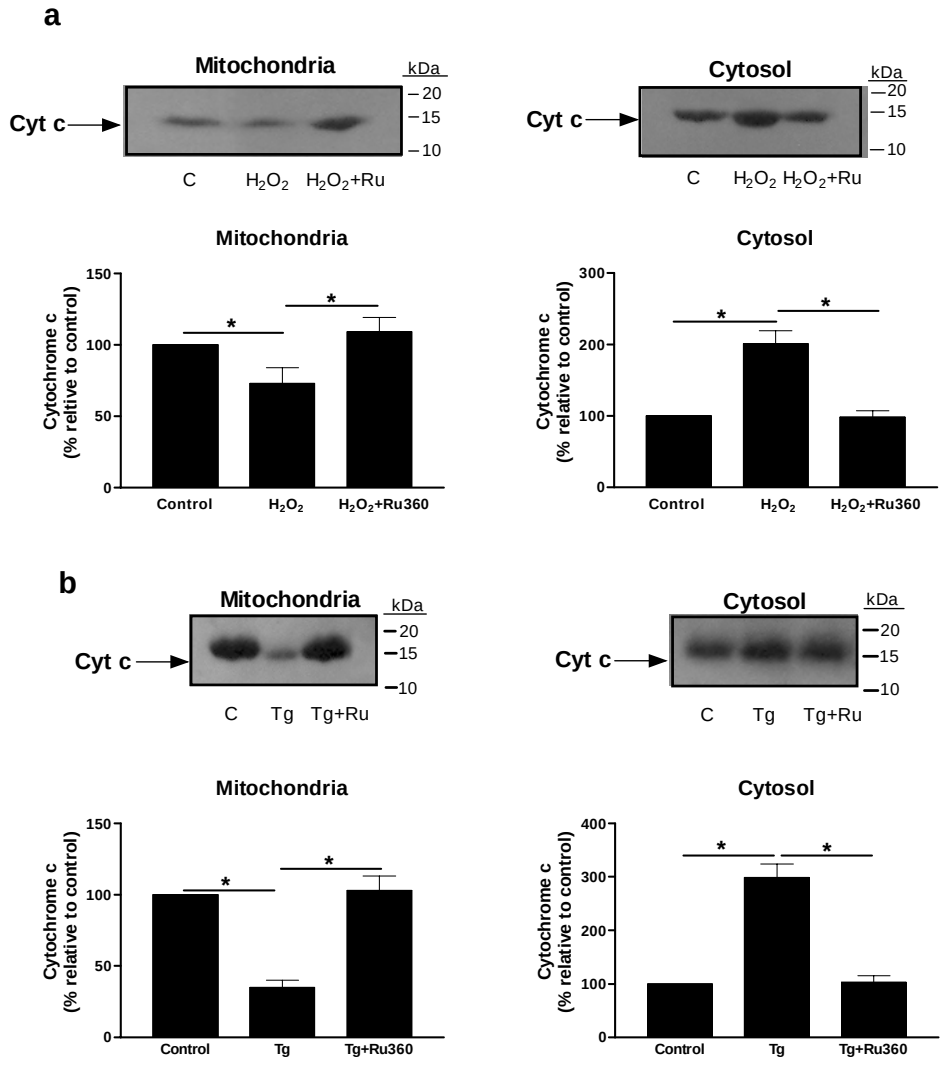

Fig. 8. **Effect of blockade of calcium uptake into mitochondria on H_2O_2-induced cytochrome c release.** Control or Ru360 (10 μM for 30 min)-pretreated AR42J cells were incubated for 24 h with H_2O_2 (10 μM) (a) or thapsigargin (Tg) (1 μM) (b). The cytochrome c content in the cytosolic and mitochondrial fractions was determined by Western blotting as described in "Material and Methods". Panels show a blot representative of three others. Histograms represent the change in cytochrome c content and is presented as a percentage relative to control (untreated samples). Values are represented as means ± S.E.M. of 3 separate experiments; *statistically significant.

cells, which undergo self-destruction by the activation of an intrinsic cell suicide program. This study was designed to determine whether calcium-mediated death signals are involved in ROS-induced cell death, since calcium and ROS have been reported to be critical apoptosis-eliciting mediators. Our studies demonstrate that H_2O_2 induces apoptotic events in rat pancreatic acinar AR42J cells, which are mostly of mitochondrial origin, such as depolarization of Ψm, cytochrome c release and caspase-3 activation; and this apoptosis was mediated by the calcium uptake into mitochondria. The blockade of mitochondrial calcium entry by pretreating with Ru360 showed protection against H_2O_2- and thapsigargin-induced apoptosis.

$[Ca^{2+}]c$ measurements showed that H_2O_2 increased $[Ca^{2+}]c$ in the presence or absence of

extracellular calcium, indicating that H_2O_2 mobilizes calcium from intracellular stores in AR42J cells. Similar results have been obtained by us in other cellular types, such as mouse pancreatic acinar cells (Pariente et al. 2001, Granados et al. 2006), human platelets (Redondo et al. 2004, Rosado et al. 2004) and neutrophils (Bejarano et al. 2007), and rat hippocampal astrocytes (González et al. 2006). Sustained $[Ca^{2+}]c$ elevation has been recognized as a trigger of apoptosis cell death (Hajnoczky et al. 2003, Nicotera and Orrenious 1998). In addition, calcium overloading in mitochondria can induce a cell suicide program by stimulating the release of apoptosis promoting factors such as cytochrome c, and by generating ROS due to respiratory chain damage (Green and Reed 1998, Wang 2001, Tan et al. 1998, Brookes et al. 2004, Hajnoczky et al. 2006). Furthermore, mitochondria have been found to play a pivotal role in calcium signalling (Hajnoczky et al. 2003).

Our study indicates that the effect of H_2O_2 on mitochondrial cytochrome c release and caspase-3 activation is not dependent on elevations in [Ca2+]c as demonstrated by loading the AR42J cells with the intracellular calcium chelator, dimethyl BAPTA. To further investigate the role of calcium on apoptosis, we used thapsigargin, which fully depletes the calcium intracellular stores. Basically, the cellular effect of thapsigargin can be summarized in calcium store depletion and elevation of $[Ca^{2+}]c$. As observed with H_2O_2, in dimethyl BAPTA-loaded cells, treatment with thapsigargin reported the same results as in the absence of dimethyl BAPTA. Therefore, since elevations in $[Ca^{2+}]c$ are not required for caspase-3 activation and mitochondrial cytochrome c release, the H_2O_2- and thapsigargin-induced cell death observed by us must depend solely on mitochondrial calcium entry. We provide compelling evidence in support of our proposal that mitochondrial calcium uptake, independently of rises in $[Ca^{2+}]c$, induces cytochrome c release and caspase-3 activation. Our results show that the blockade of calcium uptake into mitochondria with Ru360 was able to decrease the programmed cell death mediated by H_2O_2 and thapsigargin, which were able to release calcium from intracellular stores. Similar results have been obtained by Hajnoczky et al (2006), whose data confirmed that Ru360 is a potent inhibitor of the mitochondrial calcium uptake in permeabilized H9c2 rat cardiac myoblast cells. The lack of the effect of [Ca2+]c elevation on cytochrome c release and caspase-3 activation is consistent with evidence showing a direct interaction between mitochondria and intracellular calcium stores and the observation of close physical contacts between both organelles (Rizzuto et al. 1998, Csordas et al. 1999). In fact, there is both structural and functional evidence suggesting the presence of specific and stable interactions between mitochondria and intracellular calcium stores (e.g. endoplasmic reticulum) which facilitate a rapid and nearly direct flux of calcium from endoplasmic reticulum to mitochondria (Rizzuto et al. 1998, Hajnoczky et al. 2000, Camello-Almaraz et al. 2002, Filippin et al. 2003, Vay et al. 2007) and these tight endoplasmic reticulum-mitochondria couplings may also serve to modulate calcium release. In addition, it has been suggested that mitochondria colocalize in small subcellular regions where reticulum and mitochondria form close contacts (Vay et al. 2007). This has led to the concept of "intracellular synapse" or "quasi-synaptic" calcium signal transmission between mitochondria and reticulum coined by Hajnozcky's group (Csordas et al. 1999). The endoplasmic reticulum-mitochondrial interface may be supported by direct links between the organelles or by anchorage of the organelles to cytoskeletal framework (Hajnoczky et al. 2006).

Finally, the relationship between ROS and apoptosis and/or necrosis in the pathophysiology of the acute pancreatitis in acinar cells has been previously reported. In cerulein-induced pancreatitis, a high degree of ROS generation and apoptosis were observed in pancreatitis acinar cells (Kimura et al. 1998, Yu et al. 2002). In addition, ROS production in the AR42J pancreatoma cell line during the course of pancreatitis induced by cerulein was mediated by NADPH oxidase, which induces apoptosis (Yu et al. 2005); and oxidative stress, generated by H_2O_2, induced apoptosis as determined by quantification of DNA fragmentation and expression of pro- and anti-apoptotic proteins in AR42J cells (Song et al. 2003).

In conclusion, in the present investigation we have shown that the reactive oxygen specie H_2O_2 stimulates apoptosis in rat pancreatic acinar AR42J cells, which is mediated by mitochondrial depolarization and cytochrome c release, and caspase-3 activation. Similar results were obtained when the intracellular calcium stores were depleted by treatment with thapsigargin. This H_2O_2- and thapsigargin-induced programmed cell death requires calcium store depletion but is independent of rises in $[Ca^{2+}]c$, similar to a number of physiological processes including store-operated calcium entry (Rosado et al. 2005). We propose that the apoptosis evoked by H_2O_2 and thapsigargin are mediated by mitochondrial calcium uptake.

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