Apoptosis induced by exposure to a low steady-state concentration of H_2O_2 is a consequence of lysosomal rupture

Fernando ANTUNES*†[1], Enrique CADENAS* and Ulf T. BRUNK‡

*Department of Molecular Pharmacology and Toxicology, School of Pharmacy, University of Southern California, 1985 Zonal Ave., PSC-622, Los Angeles, CA 90089-9121, U.S.A., †Grupo de Bioquímica e Biologia Teóricas and Centro de Estudos de Bioquímica e Fisiologia, Instituto Bento da Rocha Cabral, P-1250 Lisboa, Portugal, and ‡Division of Pathology II, Faculty of Health Sciences, University of Linköping, Linköping, Sweden

We have re-examined the lysosomal hypothesis of oxidative-stress-induced apoptosis using a new technique for exposing cells in culture to a low steady-state concentration of H_2O_2. This steady-state technique mimics the situation *in vivo* better than the bolus-administration method. A key aspect of H_2O_2-induced apoptosis is that the apoptosis is evident only after several hours, although cells may become committed within a few minutes of exposure to this particular reactive oxygen species. In the present work, we were able to show, for the first time, several correlative links between the triggering effect of H_2O_2 and the later onset of apoptosis: (i) a short (15 min) exposure to H_2O_2 caused almost immediate, albeit limited, lysosomal rupture; (ii) early lysosomal damage, and later apoptosis, showed a similar dose-related response to H_2O_2; (iii) both events were inhibited by pre-treatment with iron chelators, including desferrioxamine. This compound is known to be taken up by endocytosis only and thus to become localized in the lysosomal compartment. After exposure to oxidative stress, when cells were again in standard culture conditions, a time-dependent continuous increase in lysosomal rupture was observed, resulting in a considerably lowered number of intact lysosomes in apoptotic cells, whereas non-apoptotic cells from the same batch of oxidative-stress-exposed cells showed mainly intact lysosomes. Taken together, our results reinforce earlier findings and strongly suggest that lysosomal rupture is an early upstream initiating event, and a consequence of intralysosomal iron-catalysed oxidative processes, when apoptosis is induced by oxidative stress.

Key words: desferrioxamine, dipyridyl, iron, Jurkat T-cell, necrosis.

INTRODUCTION

By increasing oxidative stress, a range of cellular events, which induce proliferation, growth arrest, apoptosis or necrosis, are triggered, pointing to an important physiological role of redox regulation in growth homoeostasis [1,2]. Although most current studies on oxidative-stress-induced apoptosis focus on caspases, mitochondrial energy changes and plasma-membrane-bound 'death' receptors, evidence has been brought forward for a role of lysosomes in the initiating phase of this important physiological process [3–12]. Lysosomes, which together with late endosomes constitute the acidic vacuolar apparatus, are the main cellular compartment for intracellular degradation and contain a wide spectrum of hydrolytic enzymes. Abundant normal autophagocytotic degradation of metalloproteins, such as cytochromes, ensures the intralysosomal occurrence of redox-active low-molecular-mass iron. This, along with the participation of iron in Fenton reactions to yield hydroxyl radicals ($HO^{\bullet}$), accounts for the sensitivity of lysosomes to oxidative stress that, if intense enough, may result in lysosomal rupture and ensuing cellular damage [3,4]. Although Christian de Duve, who discovered lysosomes, envisaged such a possibility by nicknaming lysosomes 'suicide bags' [13], lysosomes today are generally, although we believe wrongly, considered to be sturdy organelles that do not usually rupture until the cell is already dying and necrotic.

Both early lysosomal rupture and later apoptosis may be induced by exposing cells in culture to bolus additions of H_2O_2 [3,4]. This approach, however, disrupts cellular homoeostasis and induces severe non-physiological stress; hence, the role of lysosomes in oxidative-stress-induced apoptosis needs be confirmed using a more physiological experimental model. Moreover, because H_2O_2 added as a bolus is consumed within minutes [14], while apoptosis does not occur until hours later, any satisfactory hypothesis on the mechanisms behind oxidative-stress-induced apoptosis must provide firm and distinct links between the triggering events, which occur within minutes, and the ultimate apoptosis.

Recently, we described a new technique to expose cells to a low and steady-state concentration of H_2O_2 under otherwise standard culture conditions. This technique produces conditions that resemble those *in vivo* better than the conventional bolus-addition method [15]. Employing the former procedure, it was shown that the induction of apoptosis by H_2O_2 proceeds through Fenton chemistry and not by interfering with thiol or selenium metabolism [15], a finding that is compatible with the lysosomal hypothesis of oxidative-stress-induced apoptosis [3–7]. In addition, the steady-state method of H_2O_2 delivery makes it possible to control the period of oxidative-stress exposure, providing a new and sensitive tool to assess and correlate very short periods of exposure to oxidative stress with the ensuing onset of apoptosis.

Abbreviation used: $[H_2O_2]_{ss}$, steady-state H_2O_2 level.

[1] To whom correspondence should be addressed at current address, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal (e-mail fantunes@fc.ul.pt).

In this study, we furnish evidence for a primary role of lysosomes in H_2O_2-induced apoptosis and describe a close correlation between limited lysosomal rupture, taking place within minutes of the onset of H_2O_2 exposure, and a later developing apoptosis.

MATERIALS AND METHODS

Reagents

Acridine Orange and catalase (bovine liver) were from Fluka (Buchs, Switzerland). Glucose oxidase (*Aspergillus niger*, grade II) was from Boehringer Mannheim (Mannheim, Germany). Desferrioxamine, 2,2′-dipyridyl, diethylenetriaminepenta-acetic acid (DTPA), H_2O_2, Hepes and all other chemicals, unless otherwise stated, were from Sigma (St Louis, MO, U.S.A.).

Cell cultures and exposure to H_2O_2 with and without pre-treatment with iron chelators

Jurkat T-cells (clone E6-1) were obtained from the ATCC (Manassas, VA, U.S.A.) and cultured in complete growth medium (RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine and antibiotics; Life Technologies, Rockville, MD, U.S.A.). Cells were incubated at 37 °C in humidified air with 5% CO_2, and kept in the logarithmic growth phase by routine passage every 2–3 days. Before use, cells were centrifuged (600 ***g***, 5 min), resuspended in fresh medium at 1×10^6 cells/ml and incubated for another 2 h at standard conditions. Cells were exposed to H_2O_2 in complete medium as described in [15]. In brief, steady-state levels of H_2O_2 ($[H_2O_2]_{ss}$) were obtained by adding an initial amount of H_2O_2 together with some glucose oxidase that, by forming H_2O_2, compensated for the consumption of H_2O_2 by the cells. By balancing the initial additions of H_2O_2 and glucose oxidase, a steady-state concentration range of 0–60 μM was achieved and tried. The incubation period was controlled by adding an excess of catalase at desired points of time to reduce the H_2O_2 concentration to virtually zero. In some experiments, cells were pre-incubated for 30 min with the metal chelators desferrioxamine (1 mM) or 2,2′-dipyridyl (100 μM), under otherwise standard culture conditions, before exposure to H_2O_2.

Determination of lysosomal membrane stability

Static cytofluorimetry

Cells were exposed to the lysosomotropic weak base Acridine Orange as described previously [3–7,16]. Due to proton trapping, this vital dye accumulates mainly in the acidic vacuolar apparatus, preferentially in secondary lysosomes [3–7,17–20]. Acridine Orange is also a metachromatic fluorophore. When excited by blue light it shows red fluorescence at high (lysosomal) concentrations and green fluorescence at low (nuclear and cytosolic) concentrations [3–7,17–20]. If, however, green excitation light is used, only concentrated (lysosomal) Acridine Orange is demonstrated, by its red fluorescence [4,6,16,20]. As long as the lysosomal and cytosolic pH values are not changed drastically (increased and decreased, respectively), causing increased cytosolic accumulation of the protonated form of Acridine Orange, rupture of initially Acridine Orange-loaded lysosomes may be monitored as an increase in cytoplasmic diffuse green fluorescence, or as a decrease in granular red fluorescence [4,6,16,20].

Since most photomultipliers are more sensitive to green than to red photons, the registration of increased green cytosolic fluorescence (by excitation of Acridine Orange with blue light) is the more precise method to evaluate lysosomal burst [4,6,16,20]. Consequently, this method was utilized to reveal very early alterations, whereas the registration of declining red fluorescence (following Acridine Orange excitation with green light) was used to study later effects.

Flow cytofluorimetry

Cells (2×10^5/ml) were stained with Acridine Orange (5 μg/ml) in RPMI 1640 medium with Hepes buffer (10 mM, pH 7.3) for 15 min at 37 °C. Cells were then washed, resuspended in complete medium at 1×10^6/ml and exposed to H_2O_2. At the end of H_2O_2 exposure, cells were resuspended in PBS and the green (channel 1) and red (channel 2) fluorescence of 10^4 cells was recorded on a logarithmic scale by flow cytofluorometry using a Becton-Dickinson FACScan instrument (no band-pass filters) while excited at 488 nm (argon laser). Using this technique early alterations of lysosomal stability were assayed (see above). In other experiments, designed to study later effects, cells were stained with Acridine Orange after the H_2O_2 exposure. All steps were carried out in the dark. Results are reported as medians of the population fluorescence.

Apoptosis assays

Apoptosis was measured at the indicated times after the initiation of H_2O_2 exposure by following the flip-flop of phosphatidylserine from the inner- to the outer-plasma-membrane leaflet, using flow cytofluorimetry. A kit (Oncogene Research Products, Cambridge, MA, U.S.A.) containing propidium iodide and an FITC conjugate of annexin V was used according to the manufacturer's instructions. Morphological observation (by light microscopy, using magnifications up to $\times 1000$) was also performed, and at least 200 cells/slide were counted. The levels of apoptosis assayed by morphology (cytoplasmic budding and nuclear pycnosis or fragmentation) were similar to those obtained by registering the flip-flop of phosphatidylserine. All experiments were performed in duplicate and repeated at least three times.

RESULTS

Oxidative stress and apoptosis: the effects of time and dose

The apoptotic response of Jurkat T-cells to $[H_2O_2]_{ss}$ followed an S-shaped curve: a threshold level was reached at 5–10 μM, there was a sharp increase in apoptosis in the 10–20 μM range and saturation occurred at concentrations higher than 20 μM (Figure 1A). A striking characteristic of H_2O_2-induced apoptosis in Jurkat-T cells is that two different time scales are involved: exposure of cells to a $[H_2O_2]_{ss}$ value of 25 μM for 15 min was sufficient to induce a significant level (15%) of apoptosis 12 h later (Figure 1B), whereas exposure to the same steady-state level of H_2O_2 for 60 min required 4 h to observe similar levels of apoptosis (Figure 1C). Therefore, the results depicted in Figure 1 show that H_2O_2 can trigger a cascade of events within minutes, resulting in the onset of apoptosis hours later. No significant number of necrotic cells, having abnormal propidium iodide-stained nuclei as a consequence of abnormal plasma-membrane permeability, was found upon exposure to low $[H_2O_2]_{ss}$.

Lysosomal damage during H_2O_2-induced apoptosis

Lysosomal damage was assayed by cytofluorimetric assessment of the changes in green and red fluorescence of cells stained with Acridine Orange before or after the exposure to oxidative stress, respectively. Acridine Orange is a weak base that, due to proton

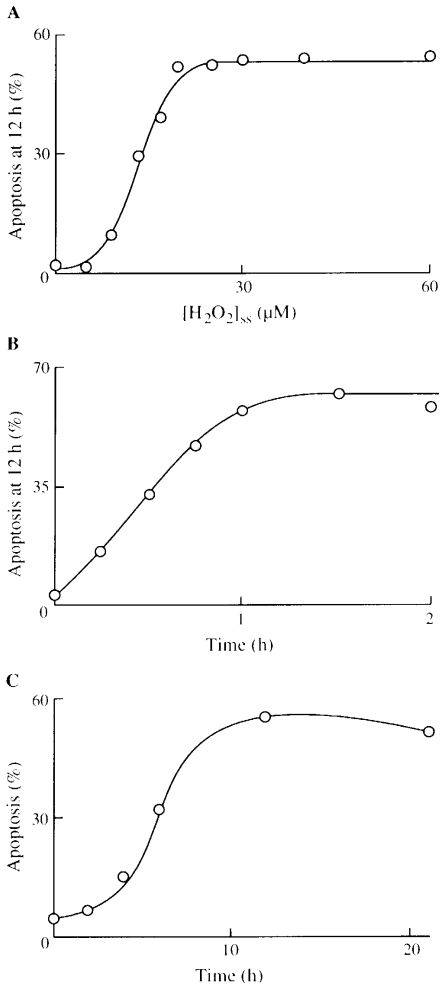

Figure 1 Induction of apoptosis by H_2O_2

Cells were exposed to different $[H_2O_2]_{ss}$ values for 60 min (**A** and **C**) or for the indicated periods (**B**), and then returned to standard culture conditions. Apoptosis was assessed by propidium iodide and annexin V staining 12 h after the start of H_2O_2 exposure (**A** and **B**), or after the indicated periods of time (**C**). In (**B**) and (**C**), cells were exposed to $[H_2O_2]_{ss} = 25\ \mu M$.

trapping, preferentially distributes within the acidic vacuolar (lysosomal) cellular compartment [3–7,17–20]. Due to its metachromatic properties, this probe fluoresces red inside the lysosomes, where it is highly concentrated, and weakly green in the cytosol and nucleus, where it is much less concentrated. When used as a vital stain at low concentrations, the intercalation of Acridine Orange into RNA and DNA is very low and does not disturb the evaluation of lysosomal stability.

In order to establish a correlation between lysosomal damage and apoptosis, red fluorescence was measured in cells with apoptotic (Figure 2A) and normal (Figure 2B) morphology from the same cell batch exposed to a $[H_2O_2]_{ss}$ of 18 μM. A good correlation was observed between cells showing a decrease of the red fluorescence ('pale' cells), which is indicative of a decrease in the number of intact lysosomes, and morphological changes typical of apoptosis following exposure to $[H_2O_2]_{ss}$. This correlation was maintained over a range of $[H_2O_2]_{ss}$ values (Figure 2C). However, these results do not provide information on whether lysosomal damage is a cause or a consequence of apoptosis. Further insight into this problem was obtained by following the time course of appearance of 'pale' cells: in order to observe a significant decrease in red fluorescence, a lag period of several hours was required (Figure 3), as was also observed when following the onset of apoptosis. It may be concluded that despite the good correlation mentioned above, the assessment of lysosomal disruption by following red fluorescence did not provide support for the hypothesis that H_2O_2 directly, and within minutes, damages lysosomes.

Most photomultipliers are about 10-fold less sensitive in the red spectral region than in the green region. Thus an increased cytosolic green fluorescence, due to Acridine Orange leak from damaged lysosomes, is a more sensitive marker of lysosomal rupture than a decline in red fluorescence. Results obtained by flow cytofluorimetry presented in Figure 4(A) show that a 15 min exposure to a saturating $[H_2O_2]_{ss}$ (25 μM) triggered significant lysosomal disruption, as assessed by the evident increase in green cytosolic fluorescence. Furthermore, the magnitude of fluorescence observed after a 60 min exposure to different H_2O_2 concentrations correlated well with the degree of ensuing apoptosis observed 12 h later (Figure 4B).

Because a certain amount of Acridine Orange is trapped inside lysosomes following exposure to this lysomotropic compound, cytosolic Acridine Orange concentration increases upon lysosomal disruption, causing enhanced green fluorescence. If staining with Acridine Orange were to be performed after exposure to H_2O_2, when some lysosomes are already disrupted, no major increase in cytosolic green fluorescence would be expected, unless there was a drastic acidification of the cytosol (that would cause some increased binding of Acridine Orange in its protonated form) due to the lysosomal rupture. No significant increase of Acridine Orange-induced cytosolic green fluorescence was observed when Acridine Orange staining was performed after H_2O_2 exposure (results not shown), thus suggesting that exposure to H_2O_2 *per se* does not change the staining properties of the cytosol. This strongly supports the notion that the increase in green fluorescence (Figure 4) is due to lysosomal release of Acridine Orange.

It is known that apoptosis is accompanied by a slight decrease in cytosolic pH; however, because the relocation of Acridine Orange to the cytosol (increased green fluorescence) occurred minutes after exposure to H_2O_2 (Figure 4A) and long before any sign of apoptosis, any relation to altered cytosolic pH can be ruled out. Taken together, the results presented in Figure 4 provide a strong correlation between early lysosomal damage and late apoptosis in cells exposed to H_2O_2.

Fenton chemistry, lysosomes and apoptosis

The involvement of lysosomes in apoptosis was examined further by assessing the effect of two metal chelators, desferrioxamine and 2,2′-dipyridyl, which protect cells effectively against H_2O_2-induced apoptosis [3,4,15]. These agents protect lysosomes against damage caused by H_2O_2, because chelation of intralysosomal redox-active transition metals is expected to prevent oxidative reactions with ensuing lysosomal rupture [3,4]. The results shown in Figure 5 indicate that these chelators partially protect lysosomes against exposure to steady-state levels of H_2O_2 for up to 60 min.

Desferrioxamine is taken up into cells by endocytosis [21–23] and, consequently, binds lysosomal low-molecular-mass redox-active transition metals. 2,2′-Dipyridyl is a lipophilic compound that transverses membranes freely [24]; hence, a broader cellular range of effects may be expected with 2,2′-dipyridyl. Because both compounds elicit the same effect (protecting against lysosomal rupture and the ensuing apoptosis), it may be surmised

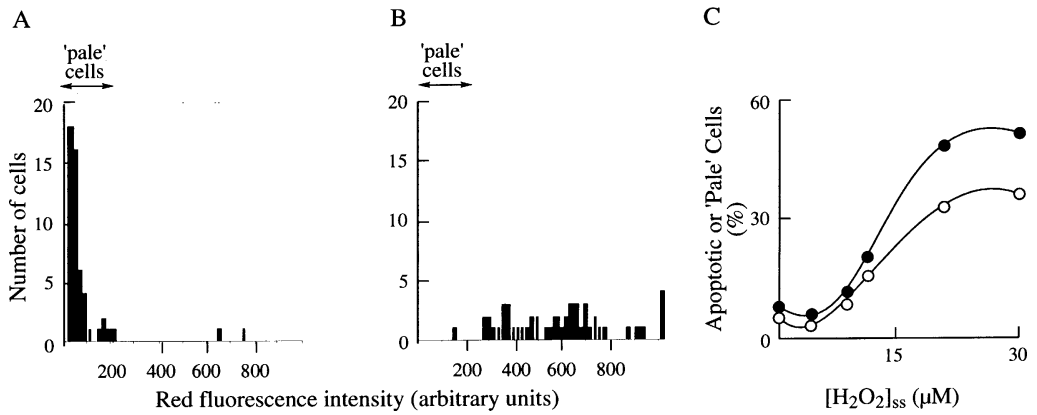

Figure 2 Apoptotic cells have few remaining intact lysosomes

Cells were exposed for 60 min to $[H_2O_2]_{ss}$, rinsed, and returned to normal conditions. After 15 h, cells were exposed to the lysosomotropic weak base and metachromatic flurophore, Acridine Orange, and red fluorescence of individual cells was measured by static cytofluorimetry. Cells with apoptotic morphology (**A**) and normal-looking cells (**B**) from the same dish exposed to 18 μM H_2O_2. (**C**) Number of 'pale' (○) and apoptotic (●) cells observed after exposure to $[H_2O_2]_{ss}$. Note an increased number of cells with less than normal red fluorescence ($\leqslant$ 200 fluorescence arbitrary units) in (**A**) (indicated as 'pale' cells in the histograms) due to lysosomal rupture.

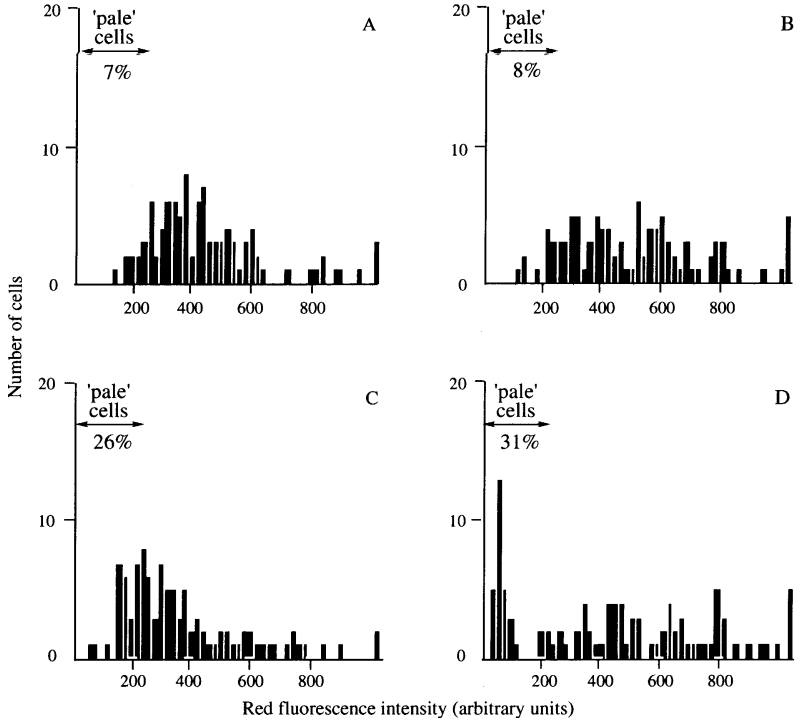

Figure 3 Disruption of lysosomes by H_2O_2

Cells were exposed for 60 min to 27 μM $[H_2O_2]_{ss}$ followed by exposure to Acridine Orange at 0 h (**A**), 1 h (**B**), 4 h (**C**) and 8 h (**D**) after the onset of H_2O_2 exposure. Fluorescence of individual cells was measured by static cytofluorimetry. Some 'pale' as well as apoptotic cells are already present under control conditions, thus reflecting the spontaneous frequency of apoptosis within this population of malignant cells.

that lysosomal transition metals, probably iron, are important in the sequence of steps triggered by H_2O_2 and leading to apoptosis.

DISCUSSION

Apoptosis is responsible for tissue homoeostasis and, therefore, must be regulated tightly. Caspases belong to a group of evolutionarily well-conserved proteases, presently comprising more than a dozen members within three main families [25]. They are believed to play a critical role in the induction and execution phases of apoptosis. In order for apoptosis to proceed, pro-caspases must be activated by proteolytic cleavage. This process may require an interplay between pro-caspases and different cell organelles and plasma-membrane-bound receptors.

In the present study, we present further evidence for the involvement of lysosomes in apoptosis, and show that oxidative-

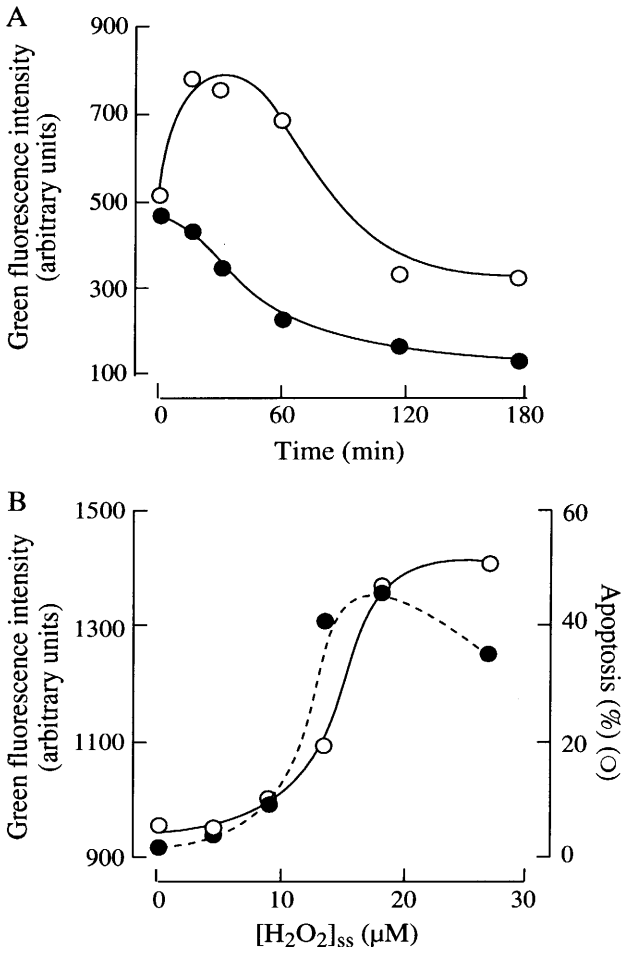

Figure 4 Time-dependent lysosomal rupture following exposure of cells to H_2O_2

Cells were pre-treated with Acridine Orange and then exposed to H_2O_2. (**A**) Green fluorescence, indicating the relocation of Acridine Orange from lysosomes to the cytosol, was measured by flow cytofluorimetry after the onset of H_2O_2 exposure (25 μM; 0–180 min); ●, control cells; ○, H_2O_2-exposed cells. (**B**) Apoptosis was assayed 12 h later by annexin binding to phosphatidylserine at the surface of the cells (flow cytofluorimetry) following exposure to various $[H_2O_2]_{ss}$ for 60 min; ○, apoptosis; ●, green fluorescence intensity. Note signs of lysosomal rupture within minutes of exposure to oxidative stress (**A**), as well as the correlation, at a given $[H_2O_2]_{ss}$ value, between apoptosis (○) at 12 h and the estimated lysosomal rupture (●) at the end of the 60 min period of H_2O_2 exposure (**B**).

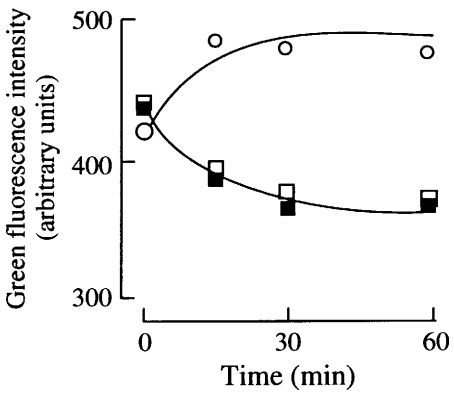

Figure 5 Effects of desferrioxamine and 2,2′-dipyridyl on lysosomal rupture following exposure of cells to H_2O_2

Cells were pre-incubated for 30 min with desferrioxamine (1 mM) or 2,2′-dipyridyl (100 μM), stained with Acridine Orange, resuspended in fresh medium and finally exposed to H_2O_2 (25 μM). Samples were assayed by flow cytofluorimetry, and green fluorescence was measured. ○, Cells exposed to H_2O_2; □ and ■, cells exposed to H_2O_2 in the presence of desferrioxamine or 2,2′-dipyridyl, respectively.

stress-related apoptosis in human Jurkat T-cells involves early partial rupture of the acidic vacuolar apparatus. The lysosomal compartment is the main arena for degradative activities of the cell, at least as far as long-lived proteins and cellular organelles are concerned [26]. Consequently, lysosomes contain a throng of powerful hydrolytic enzymes, although the composition may differ substantially between different types of cells. As mentioned above, currently lysosomes are mostly considered to be sturdy organelles that break only late during necrotic (accidental) cell death. However, the use of our very sensitive Acridine Orange-relocalization method for the detection of lysosomal destabilization has made it possible to monitor the rupture of only a few lysosomes within living cells. Using this powerful technique, we have presented evidence previously for a large variability, both within and between cells, with respect to lysosomal stability under oxidative stress [3]. The sensitivity of lysosomes to oxidative stress may be explained in several ways. Normal autophagocytotic degradation of metalloproteins, such as mitochondrial cytochromes, cause the lysosomal apparatus to temporarily harbour a considerable amount of low-molecular-mass iron before this transition element is transferred to the cytosol, where it is used by the anabolic machinery or stored in ferritin [27]. Further, the lysosomal interior is acidic, which not only facilitates iron release from proteins, but also enhances the oxidative damage caused by iron [28]. Lysosomes also maintain a high concentration of the amino acid cysteine [29], which may participate in the reduction of iron:

$$\text{Cys-SH} + \text{Fe}^{3+} \rightarrow \text{Cys-S}^{\bullet} + \text{Fe}^{2+} + \text{H}^{+}$$

In the case of oxidative stress, H_2O_2 may access the lysosomal compartment and, thus,the stage would be set for intralysosomal Fenton-type reactions, which, if violent enough, may result in attack on lysosomal membranes with ensuing peroxidation and destabilization. Differences in lysosomal stability against oxidative stress within and between cells perhaps reflect differences in their capacity to degrade H_2O_2 and/or lysosomal amounts of reactive low-molecular-mass iron.

In the present work, by showing that partial rupture may occur also under low $[H_2O_2]_{ss}$, we confirmed and extended previous results, indicating that lysosomes are very sensitive to oxidative stress. The application of steady-state oxidative stress avoids the exposure of cells to initial high and disruptive levels of H_2O_2, as occurs with the usual method of adding H_2O_2 to cell-culture media in bolus doses. A cell-surrounding concentration as low as 10 μM $[H_2O_2]_{ss}$ for 1 h induced significant lysosomal rupture. The actual concentration of H_2O_2 sensed by the lysosomes is, however, probably lower than 2 μM, because a steep gradient across the plasma membrane is established when Jurkat T-cells are exposed to H_2O_2 [14].

The finding that exposure to two structurally unrelated metal chelators preserved lysosomal stability both under and after the steady-state oxidative stress, and prevented apoptosis as well, is consistent with our previous observations when H_2O_2 was used in large bolus doses [3,4]. The protection by pre-exposure to desferrioxamine, which is taken up only by endocytosis and stored intralysosomally [21–23], confirms that lysosomal vul-

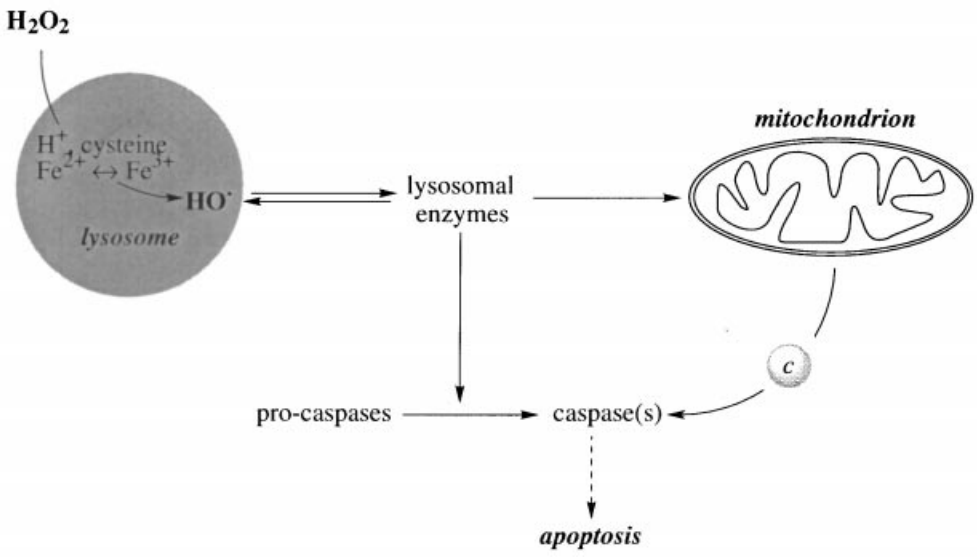

Figure 6 The lysosomal-mitochondrial hypothesis of oxidative-stress-induced apoptosis

The scheme shows how intralysosomal Fenton-type reactions follow upon increased oxidative stress, resulting in destabilization of lysosomal membranes and release of lysosomal hydrolytic enzymes into the cytosol. Some of these enzymes may directly activate pro-caspases, while others may induce such activation by attacking mitochondria, causing release of cytochrome *c* (*c*) or by activating cytosolic degradative pro-enzymes which in turn attack mitochondria and lysosomes, causing a self-progressing cascade effect.

nerability under oxidative stress is a function of intralysosomal iron-catalysed oxidation.

A key aspect of oxidative regulation of physiological processes is the disparity of the time scales involved. The apoptotic process takes several hours to develop fully, although, as shown here, cells need be exposed to H_2O_2 for only a few minutes to be committed to apoptosis. Combining a very sensitive technique to detect lysosomal rupture with a method to deliver H_2O_2 that mimics physiological conditions, we were able to observe, for the first time, a strong correlation between a H_2O_2-induced cellular modification that occurred within minutes, partial lysosomal rupture, and the onset of apoptosis hours later. Both processes showed the same dose-dependent response to H_2O_2 and were inhibited by iron chelation.

The observations in this study, and those in previous publications, provide information on the mechanism by which H_2O_2 triggers apoptosis. Release of lysosomal contents initiates a process that results in mitochondrial destabilization as well as further lysosomal rupture (Figure 6). In this context, it is worth mentioning that the release of cytochrome *c* from Jurkat T-cell mitochondria does not occur until 1–2 h after cessation of exposure to H_2O_2 [30]. Interestingly, we observed a progressive decrease in the number of lysosomes over time, even when cells were no longer under oxidative stress. In support of our recent finding linking lysosomal rupture to apoptosis, we have recently reported that Bcl-2-overexpressing J774 apoptosis-resistant cells contained lysosomes that were significantly stabilized against oxidative stress [31]. This finding suggests that the early release of lysosomal enzymes may activate feedback processes that cause further lysosomal rupture. Such feedback processes may be an attack from the outside of released lysosomal enzymes and/or activation of lytic cytosolic pro-enzymes.

Taken together, the findings of this study suggest that limited lysosomal rupture, with ensuing relocalization of lysosomal enzymes, may induce apoptosis and be of special importance in oxidative-stress-induced apoptosis. This notion is strengthened further by the observation that inhibitors of lysosomal cathepsins, such as E-64 and pepstatin, prevent apoptosis following induced lysosomal rupture [32,33]. The details of the mechanism need to be addressed further, although the finding that cytochrome *c* is released from mitochondria not earlier than 1–2 h after H_2O_2 exposure [30] suggests that lysosomal enzymes, or other enzymes activated by the lysosomal ones, may attack mitochondrial membranes.

Cytochrome *c* is well known to induce apoptosis by forming a complex with other apoptosis-inducing factors; when activated, the latter protease cleaves pro-caspase 3, the most important of the executioner caspases, and apoptosis begins. Recent reports also point to the possibility that lysosomal cysteine proteases, such as cathepsins B and L, may by themselves activate pro-caspases and turn on the caspase cascade [8–10]. These different pathways probably act in concert, both contributing to lysosomal destabilization as an important upstream event during oxidative-stress-induced apoptosis.

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