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A cytosolic source of calcium unveiled by hydrogen peroxide with relevance for epithelial cell death

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Abstract

Oxidative stress releases intracellular calcium, which plays a pathogenic role in mammalian cell death. Here we report a search for the source of oxidative calcium in HeLa cells based on confocal epifluorescence microscopy. H_2O_2 caused a rapid increase in cytosolic calcium, which was followed by mitochondrial Ca^{2+} loading. Combined mitochondrial uncoupling with full depletion of thapsigargin-sensitive stores abrogated inositol 1,4,5-trisphosphate-mediated calcium release but failed to inhibit H_2O_2-induced calcium release, observation that was confirmed in MDCK cells. Prevention of peroxide-induced acidification with a pH clamp was also ineffective, discarding a role for endosomal/lysosomal Ca^{2+}/H^+ exchange. Lysosomal integrity was not affected by H_2O_2. Mature human erythrocytes also reacted to peroxide by releasing intracellular calcium, thus directly demonstrating the cytosolic source. Glutathione depletion markedly sensitized cells to H_2O_2, an effect opposite to that achieved by DTT. Iron chelation was ineffective. In summary, our results show the existence of a previously unrecognized sulfhydryl-sensitive source of pathogenic calcium in the cytosol of mammalian cells.

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Abbreviations: ER, endoplasmic reticulum; $InsP_3$, inositol 1,4,5-trisphosphate; BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)-ethane-*N,N,N,N*-tetraacetic acid tetra-(acetoxymethyl)-ester; BSO, L-buthionine-[*S,R*]-sulfoximine; LDH, lactate dehydrogenase; KRH, Krebs–Ringer–HEPES buffer; Glc, glucose; BCECF, 2′7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; TMRM, tetramethylrhodamine methyl ester; ROIs, regions of interest

Introduction

Supraphysiological concentrations of reactive oxygen species cause tissue damage during ischemia/reperfusion, infectious diseases and other pronecrotic conditions. One of the earliest responses to severe oxidative stress is a stereotyped increase in cytosolic calcium, which in seconds rises to a plateau at 2–3 times the resting level, a metastable condition that lasts minutes to hours. Later on, a secondary unregulated phase carries cytosolic calcium over μM levels, accompanied by sodium influx, necrotic volume increase and cell necrosis.[1,2] Milder insults can be followed by reversible functional deficits such as myocardial stunning[3] or cell death by apoptosis.[4,5] The early phase of calcium overload is important for cell death as its suppression by increasing calcium buffering greatly reduced DNA degradation,[6] mitochondrial damage,[6–9] activation of surface nonselective cation channels,[1,10] apoptosis[5,6,9] and necrosis.[6–8,10,11]

Despite its pathophysiological relevance, the source of oxidative calcium has proved elusive. Whereas all studies in non-neuronal cells have discarded an extracellular origin,[1,12–14] the participation of specific calcium storage compartments is unclear.[15] For instance, in smooth muscle cells, the calcium release was inhibited by the previous discharge of inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive stores with vasopressin.[12] In contrast, in insulin-secreting cells the calcium rise was not affected by previous depletion of thapsigargin-sensitive stores.[1] In pancreatic acinar cells, it was concluded that calcium is released from both thapsigargin-sensitive stores and the mitochondria. In that study, the thiol-reducing reagent DTT effectively blocked the calcium increase, and the $InsP_3$ receptor antagonist xestospongin C was ineffective.[13] Based on the inhibitory effect of the mitochondrial Na^+/Ca^{2+} exchanger blocker clonazepam, a recent report on PC12 cells advanced that most calcium may be released from the mitochondria, but no measurements of mitochondrial calcium/potential were carried out.[16] Possible explanations for such disparity of conclusions may be found in the use of distinct cell types and different experimental reagents/conditions. More crucial in our opinion is that these studies relied only on cytosolic calcium, with no direct estimation of relevant parameters such as mitochondrial calcium, mitochondrial potential, cytosolic pH and the filling state of intracellular calcium stores.

Here we report a search for the source of oxidative calcium in HeLa cells, using fluorescent probes to estimate calcium changes in the cytosol, the mitochondria and the endoplasmic reticulum (ER), and to estimate mitochondrial potential and cytosolic pH. We have also addressed the possible role of endosomes and lysosomes as calcium sources during oxidative stress. Our results show that a necrotic dose of H_2O_2 releases calcium mainly from the cytosol, with the mitochondria behaving as a calcium sink. The main conclusion was confirmed in a second epithelial cell line, MDCK, and also in mature human erythrocytes, which provided with a

system naturally devoid of intracellular organelles. Significantly, experiments in glutathione-depleted cells showed that the phenomenon of cytosolic calcium release can be triggered by H_2O_2 concentrations below those achieved by physiological intracellular signaling. Part of this work was presented in abstract form ('Apoptosis: from signaling pathways to therapeutic tools' meeting, Luxembourg, January 2003).

Results

Inhibition of peroxide-induced calcium release is protective

HeLa cells were chosen for this study as they are the best characterized epithelial cell type regarding calcium homeostasis, in particular with respect to the interplay between cytosol, the mitochondria and the ER.[17–21] In addition, both necrotic and apoptotic modes of cell death have been well characterized in these cells. Hydrogen peroxide was chosen as necrotic inducer due to its natural occurrence in tissues during ischemia/reperfusion, bacterial infection and inflammation, and the large body of literature accumulated on its mechanisms of production and scavenging. We have recently reported that in HeLa cells induction of necrotic changes in time frames similar to those observed in acute human pathologies, including early calcium rise (about 1 min), ATP depletion (about 1 min), necrotic bleb formation (about 1 h) and membrane permeabilization (about 2 h), was best achieved with 32 mM hydrogen peroxide.[22] A similar concentration of peroxide has been used to induce necrosis in Jurkat cells.[23] This relative resistance to oxidative stress is shared by many other cell lines,[1,24–27] a property related to their high content of reduced glutathione and enhanced flux through the pentose phosphate pathway.[26–28] A series of control experiments are described in Materials and Methods that discard possible interference between peroxide and the various fluorescent dyes used.

Exposure of HeLa cells to hydrogen peroxide caused a typical two-fold increase in cytosolic calcium, which started immediately and reached a plateau after 1–2 min (Figure 1a). The injury was followed later on by cell necrosis, which was quantified by the release of the cytosolic enzyme lactate dehydrogenase (LDH) to the medium (Figure 1b). Figure 1a also shows that the early calcium release was strongly inhibited by previous loading of the cells with the fast calcium chelator BAPTA in its ester form 1,2-bis-(*o*-aminophenoxy)-ethane-*N,N,N,N*-tetraacetic acid tetra-(acetoxymethyl)-ester (BAPTA-AM). For three separate experiments with BAPTA-loaded cells, the average increase in Rhod-2 signal after 10 min exposure to H_2O_2 was only $9 \pm 4\%$. Chelation of intracellular calcium protected against subsequent cell necrosis (Figure 1b), which confirms its pathogenic relevance. It has been shown before that BAPTA does not behave as an H_2O_2 scavenger.[11]

Endoplasmic reticulum/extracellular space

To ascertain the role of the ER and other $InsP_3$-sensitive stores as possible peroxide-sensitive calcium sources, the stores were fully depleted of calcium using a stringent protocol previously validated in HeLa cells by ER-targeted low-affinity aequorin.[29] In our hands, this protocol resulted in a significant decline in the concentration of free calcium in intracellular stores as estimated with the low-affinity probe Calcium Orange 5N (Figure 2a and b), but still caused no inhibition of the response to peroxide (Figure 2c). After 10 min exposure, the rise in calcium was $300 \pm 60\%$ (three experiments) for depleted cells, as compared with $130 \pm 20\%$ for a control set of five experiments. Therefore, the peroxide-sensitive calcium source is not to be found primarily in the ER or any other thapsigargin-sensitive store. As this experiment was performed in a buffer without added calcium plus 0.5 mM EGTA, it also serves to discard extracellular calcium as a possible source for the overload.

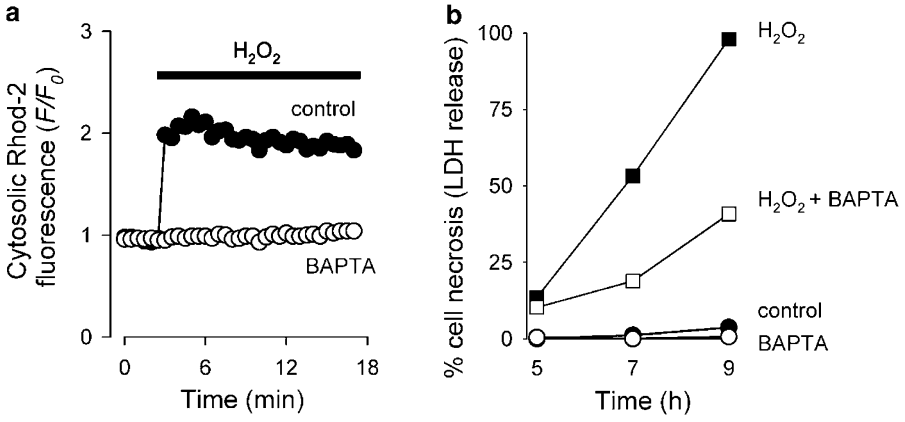

Figure 1 Intracellular BAPTA inhibits the early increase in cytosolic calcium induced by H_2O_2 and protects against subsequent HeLa cell necrosis. (**a**) The typical increase in calcium fluorescence in response to 32 mM peroxide (closed circles) was inhibited by preincubating the cells for 30 min with 75 μM BAPTA (open circles). Data are means of eight cells, representative of three separate experiments. (**b**) Control (closed squares) and BAPTA-loaded cells (open squares) were exposed to 32 mM hydrogen peroxide for 5 min. Cells were then washed and incubated for the times shown in KRH-glc prior to LDH release determination. Similar measurements with untreated cells in the absence (closed circles) and presence of BAPTA (open circles) are shown. Data are means of two 35 mm wells, each containing approximately 3×10^5 cells, representative of three separate experiments

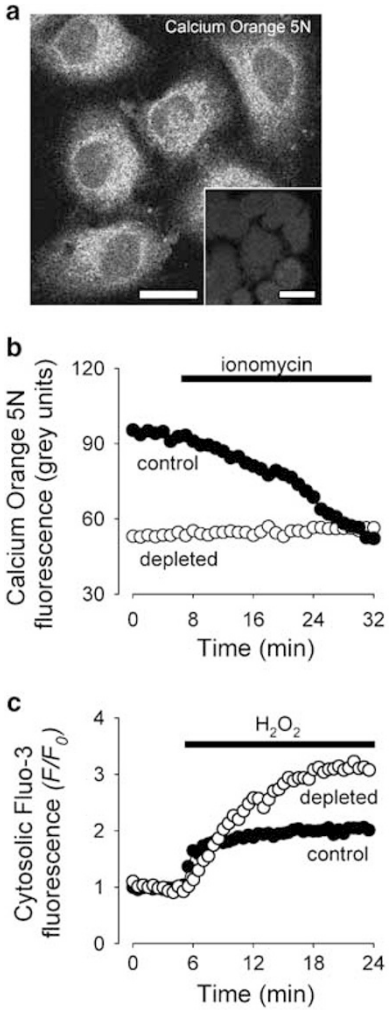

Figure 2 Full depletion of thapsigargin-sensitive calcium stores does not prevent H_2O_2-induced calcium release in HeLa cells. (**a**) Subcellular distribution of Calcium Orange 5N fluorescence before and after (inset) full depletion of calcium stores. Size bars: 25 μm. (**b**) Lack of effect to 15 μM ionomycin in calcium store-depleted cells (open circles) as opposed to nondepleted cells (closed circles). (**c**) The increase in cytosolic calcium in response to peroxide is compared in cells depleted of calcium (open circles) *versus* control cells (closed circles). For full depletion of thapsigargin-sensitive stores, cells were incubated for 5 min at 37°C with 5 μM thapsigargin in KRH-glc prepared without $CaCl_2$ and supplemented with 3 mM EGTA. Cells were then incubated for 1 h at room temperature in the same buffer with 5 μM thapsigargin and 0.5 mM EGTA. All the experiments were carried out in a nominally calcium-free medium. Data are means of 8–10 cells. Similar results were obtained in three experiments

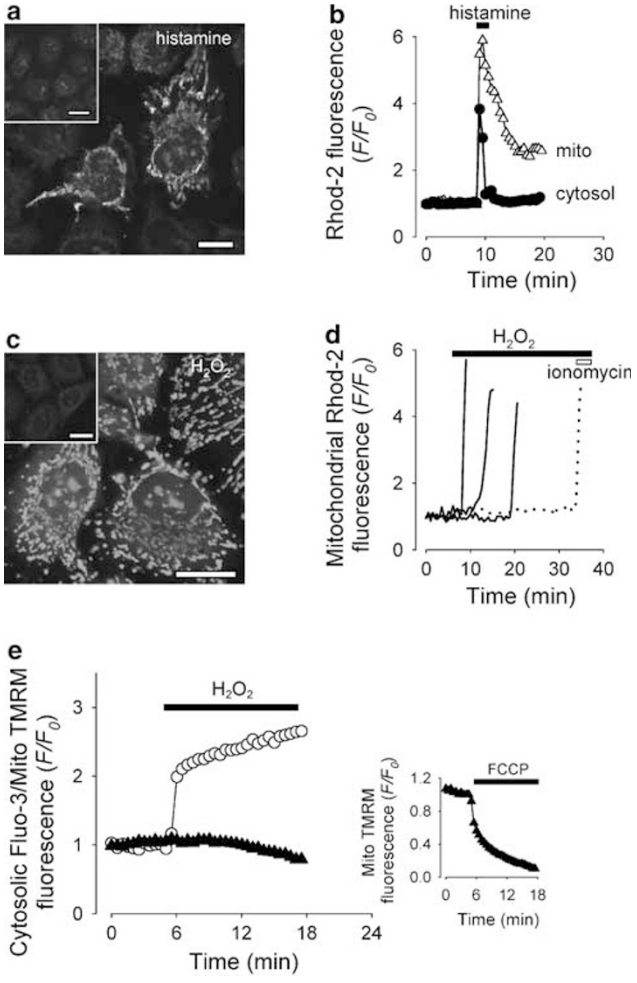

Figure 3 Mitochondrial calcium loading follows H_2O_2-induced cytosolic calcium increase in HeLa cells. Cytosolic and mitochondrial calcium fluorescence was imaged simultaneously with Rhod-2 as described in Materials and Methods. (**a**,**b**) A brief pulse of histamine induced a transient increase of calcium in the cytosol (closed circles) and a more sustained increase in the mitochondria (open triangles). Data are means of 8–10 cells. (**c**,**d**) In response to peroxide, mitochondrial calcium also increased abruptly but the response was delayed. Individual cells markedly differed as illustrated in (**d**) for three cells, with delays between 1 and 15 min. A rare case is also shown in which mitochondria did not load calcium for 30 min responding only after 2 μM ionomycin was added (dotted line). Size bars: 20 μm. (**e**) Cytosolic calcium and mitochondrial potential were assessed simultaneously with Fluo-3 and TMRM, respectively, as described in Materials and Methods. Note that mitochondrial depolarization occurs after cytosolic calcium increase. The inset illustrates the rapid decrease in mitochondrial TMRM fluorescence induced by 2 μM FCCP. Data are means of 8–10 cells. Similar results were obtained in three separate experiments

Mitochondria

These organelles can store up to 100 μM free calcium,[21] liable of sudden release should the mitochondrial potential be dissipated.[30] Two separate studies have provided inhibitor-based evidence for a mitochondrial source in H_2O_2-induced calcium rise.[13,16] In order to test this possibility more directly, we first estimated mitochondrial free calcium using the property of Rhod-2 to concentrate therein thanks to its net positive charge.[17] The ability of Rhod-2 to detect changes in mitochondrial calcium under our experimental conditions was verified with the $InsP_3$ agonist histamine (Figure 3a and b).[17] Figure 3c and d shows that in peroxide-exposed cells, a lag phase was followed by a rapid regenerative increase in mitochondrial calcium. The timing of this phenomenon was variable, occurring between 1 and 30 min after stimulation. Estimation of mitochondrial potential using the Nerstian probe tetramethylrhodamine methyl ester (TMRM) showed that H_2O_2 caused a slow depolarization of mitochondria that started well after the beginning of the cytosolic calcium increase (Figure 3e). Note the much faster depolarization induced by the protonophore FCCP (inset). These results demonstrate that in the first minutes of oxidative stress, mitochondria behave as net calcium sinks, thus ruling out these organelles as putative sources.

Next, HeLa cells and also MDCK cells were exposed to the protocol of ER depletion in the presence of FCCP, conditions under which both organelles are rendered unable to mobilize calcium. In these cells, histamine and carbachol were ineffective (Figure 4a and c) indicating that the treatment had also emptied the Golgi apparatus, another $InsP_3$-sensitive organelle.[18] In contrast, hydrogen peroxide was effective at releasing intracellular calcium in both cell types, confirming that the source of oxidative calcium in these cells is not the ER, nor the mitochondria, nor the Golgi apparatus. This conclusion was confirmed with the ratiometric probe Fura-2 (Figure 4b). Because Fura-2 ratio reports absolute calcium, the experiment also shows that resting calcium concentration in store-depleted cells was approximately half that of control cells. Therefore, the higher fold increase in fluorescence recorded by single-peak probes (Figures 2c and 4a) should only be considered as relative, and the amount of free calcium accumulated in the cytosol appears to be similar in control and store-depleted cells.

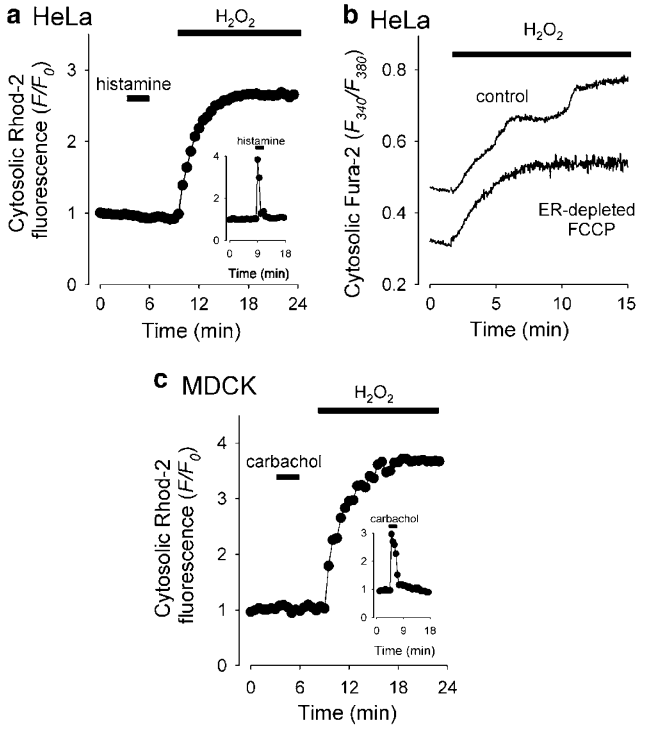

Figure 4 H_2O_2 causes intracellular calcium release in calcium-depleted HeLa and MDCK cells in the presence of a mitochondrial uncoupler. (**a**) HeLa cells were depleted of stored calcium in the absence of extracellular calcium as in Figure 2, and exposed to 2 μM FCCP for 30 min before the application of 100 μM histamine. After washing the agonist, 32 mM hydrogen peroxide was added. The inset shows that nontreated control cells responded well to histamine. Data are means of 8–10 cells, representative of three separate experiments. (**b**) Cytosolic calcium was assessed using the ratiometric dye Fura-2. The effect of 32 mM peroxide was compared in 13 ER-depleted/ FCCP-treated cells *versus* 15 control HeLa cells. Similar data were obtained in other three experiments. (**c**) MDCK cells were depleted of stored calcium and exposed to 2 μM FCCP for 30 min before the application of 100 μM carbachol. After washing the agonist, 32 mM hydrogen peroxide was added. The inset shows that nontreated control cells responded well to carbachol. Data are means of 8–10 cells, representative of three separate experiments

Acidic compartments

Other intracellular compartments rich in free calcium and therefore possible sources for the divalent are endosomes[31] and lysosomes.[32] Calcium in the endosomal network is highly dependent on continuous bulk phase endocytosis of extracellular fluid,[31] so long-time exposure to EGTA such as that used herein to deplete the ER should have caused considerable endosomal depletion too. In this light, the response to peroxide presented in Figures 2 and 4 provides indirect evidence against an endosomal source. Lysosomal calcium, which has been recently measured at about 500 μM, is much less sensitive to extracellular calcium depletion.[32] Similar to endosomal calcium, lysosomal calcium varies reciprocally with organelle proton concentration; so the large cytosolic acidification resulting from peroxide exposure[25] may in principle release the divalent in exchange for protons. An important source of protons in this case is the hydrolysis of ATP due to glycolytic inhibition by peroxide.[25] In HeLa cells coloaded with 2′7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and Rhod-2 (Figure 5a), exposure to H_2O_2 caused a significant decrease in pH, which started simultaneously with the calcium increase (Figure 5c). In order to investigate a possible role for protons as calcium agonists, we inhibited the acidification induced by peroxide by applying a

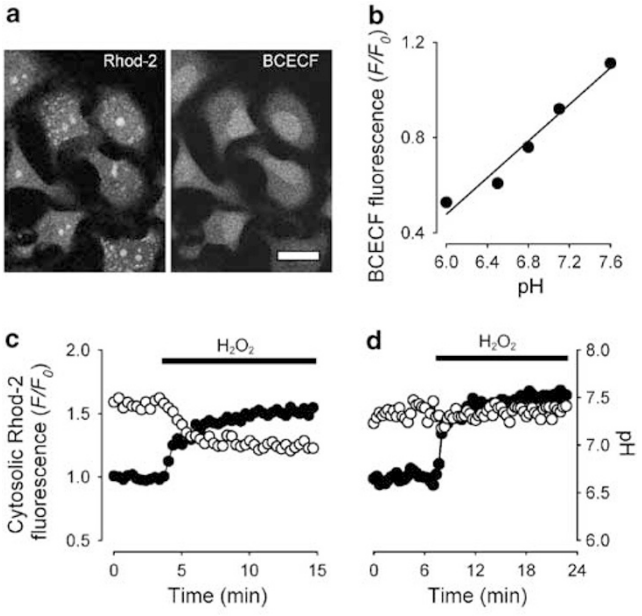

Figure 5 Intracellular acidification does not explain H_2O_2-induced cytosolic calcium increase in HeLa cells. Cytosolic calcium and intracellular pH were assessed simultaneously with Rhod-2 and BCECF as described in Materials and Methods. (**a**) Distinct patterns of Rhod-2 and BCECF fluorescence demonstrating adequate separation of the signals. Size bar: 25 μm. (**b**) BCECF was calibrated at increasing pH values in the presence of 100 μM nigericin and 10 μM gramicidin as described in Materials and Methods: BCECF fluorescence (F/F_0) = 0.38 × pH − 1.83, r = 0.98. A similar result was obtained in a separate experiment. (**c**) Simultaneous decrease in pH (open circles) and increase in calcium (closed circles) upon peroxide exposure. (**d**) Abrogation of H_2O_2-induced acidification with the pH clamp (open circles) does not eliminate the increase in cytosolic calcium (closed circles). Data are means of 8–10 cells. Similar results were obtained in three separate experiments

nigericin/gramicidin clamp. Nigericin is an H^+/K^+ exchanger that clamps proton concentration to the reciprocal of the ratio between intracellular and extracellular potassium concentration. The ratio was fixed at 1 by bathing the cells in a potassium-rich medium in the presence of the cation ionophore gramicidine. Figure 5d shows that the pH clamp efficiently inhibited the acidification, but the rapid kinetics of calcium release were unaffected. For three separate experiments, the average calcium increase after 10 min exposure to peroxide was $70 \pm 20\%$, a value similar to that of $80 \pm 20\%$ measured in nonclamped cells.

Long-term exposure to hydrogen peroxide can disrupt lysosomal membranes,[33] with a nonspecific, irreversible release of calcium that would not be inhibited by the pH clamp. In order to assess directly this possibility, HeLa cells were loaded with Lucifer Yellow, a dye that after being endocytosed becomes concentrated in lysosomes and can be released by apoptotic inducers.[34] Correct loading of the lysosomes was confirmed in our cells by high degree colocalization with the marker LysoTracker Red (Figure 6a).

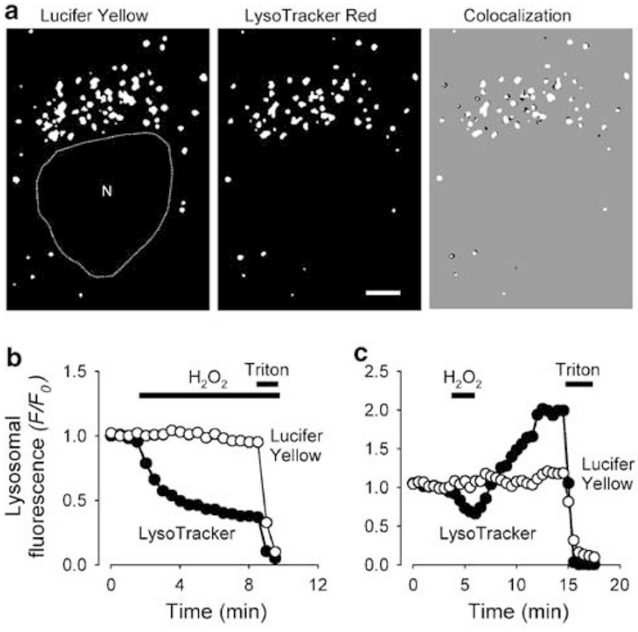

Figure 6 Lysosomes remain intact during peroxide-induced cytosolic calcium increase in HeLa cells. Lysosomes were coloaded with Lucifer Yellow and LysoTracker Red as described in Materials and Methods. (**a**) Confocal images corresponding to both dyes after filtering are shown. The panel on the right illustrates the colocalization of the signals. Regions stained with both Lucifer Yellow and LysoTracker are shown in white; regions where only LysoTracker was found are shown in black; the gray background indicates lack of staining with either dye. Image analysis indicated that 93% of the area that stained with LysoTracker was also stained with Lucifer Yellow. The dotted line indicates the nuclear contour (N). Size bar: 5 μm. Image representative of at least five experiments. (**b**) During exposure to 32 mM peroxide, Lucifer Yellow remained in lysosomes while LysoTracker Red fluorescence decreased. Both dyes were efficiently released after lysosomal rupture with 0.5% Triton X-100. Data are from four clusters of organelles, representative of more than three separate experiments. (**c**) Cells were exposed to peroxide for 2 min and then washed twice in the presence of LysoTracker. Note that while Lucifer Yellow remained stable, LysoTracker staining recovered after peroxide withdrawal. Dyes were then released with the detergent. Data are from three clusters of lysosomes. Similar data were obtained in three separate experiments

The time course in Figure 6b shows that hydrogen peroxide did not release Lucifer Yellow from lysosomes in HeLa cells. In contrast, the pH-sensitive LysoTracker was released slowly, consistent with lysosomal alkalinization as reported previously in peroxide-treated cells.[35] Upon withdrawal of the stimulus, lysosomes quickly recaptured LysoTracker, further indication of the absence of membrane disruption under these conditions (Figure 6c). Lysosomal integrity in the presence of peroxide was also confirmed in ER-depleted/FCCP-treated HeLa cells (not shown).

Cytosol

Having discarded the obvious membranous calcium stores, the possibility became apparent that calcium was being released from the large pool of divalent normally bound to cytosolic moieties. To test such idea, we investigated the capability of H_2O_2 to release calcium in a cell type devoid of organelles, namely the mature human erythrocyte. Figure 7a–c shows that exposure of erythrocytes to peroxide in the absence of extracellular calcium caused a rapid and robust increase in cytosolic calcium, demonstrating again that organelles are not required for the phenomenon. A similar response to peroxide was measured in normal extracellular calcium (not shown), indicating that in erythrocytes, as in epithelial cells, calcium influx is not significant either.

Sulfhydryl groups mediate H_2O_2-induced cytosolic calcium release

The above data show that hydrogen peroxide at high doses is capable of releasing calcium from cytosolic components in cultured cells and erythrocytes. However, these cells are known to accumulate high concentrations of glutathione, the endogenous tripeptide largely responsible for maintaining thiol groups in proteins in the reduced form. It was therefore possible that this antioxidant was counteracting the effect of peroxide, explaining the high doses required to induce calcium release and cell death. To test directly this possibility, HeLa cells were depleted of glutathione by specific inhibition of γ-glutamyl-cysteine synthase with L-buthionine-[*S,R*]-sulfoximine (BSO). The dose–response curve illustrated in Figure 8a shows that a 24 h exposure to 200 μM BSO induced 90% depletion of glutathione content, which resulted in a marked sensitization of HeLa cells, with peroxide now triggering detectable calcium increases at concentrations as low as 125 μM (Figure 8b and c). Consistent with a pathogenic role for calcium demonstrated in Figure 1, glutathione depletion also sensitized HeLa cells to peroxide-induced necrosis (Figure 8d). As a direct way to test for thiol group involvement, calcium was measured in the presence of the strong reducing agent DTT. Figure 9a and b shows that both in HeLa cells and erythrocytes, DTT effectively inhibited H_2O_2-induced calcium increase. In three separate experiments, 5 min exposure of HeLa cells to peroxide caused an 80 ± 10 and $10 \pm 10\%$ increase in cytosolic calcium in the absence and presence of DTT, respectively ($P < 0.05$). In a similar number of experiments in erythrocytes, the increases in cytosolic calcium in the absence and presence of DTT were 170 ± 20

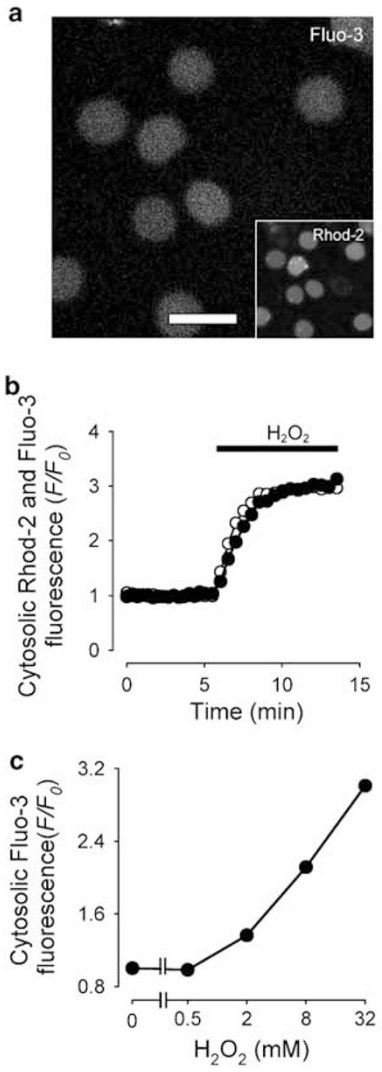

Figure 7 Hydrogen peroxide is able to release cytosolic calcium in human erythrocytes. Cytosolic calcium was assessed simultaneously with Fluo-3 and Rhod-2, as described in Materials and Methods. (**a**) Loading patterns of Fluo-3 and Rhod-2 (inset) probes in human erythrocytes. Size bar: 10 μm. (**b**) Exposure to hydrogen peroxide in nominally calcium-free KRH-glc induces a rapid increase in calcium as measured with both Fluo-3 (open circles) and Rhod-2 (closed circles). A similar result was obtained in at least three separate experiments. (**c**) Erythrocytes resuspended in KRH-glc were exposed for 10 min to increasing concentrations of hydrogen peroxide before recording the relative increase in fluorescence (closed circles). Date are means of 8–10 cells

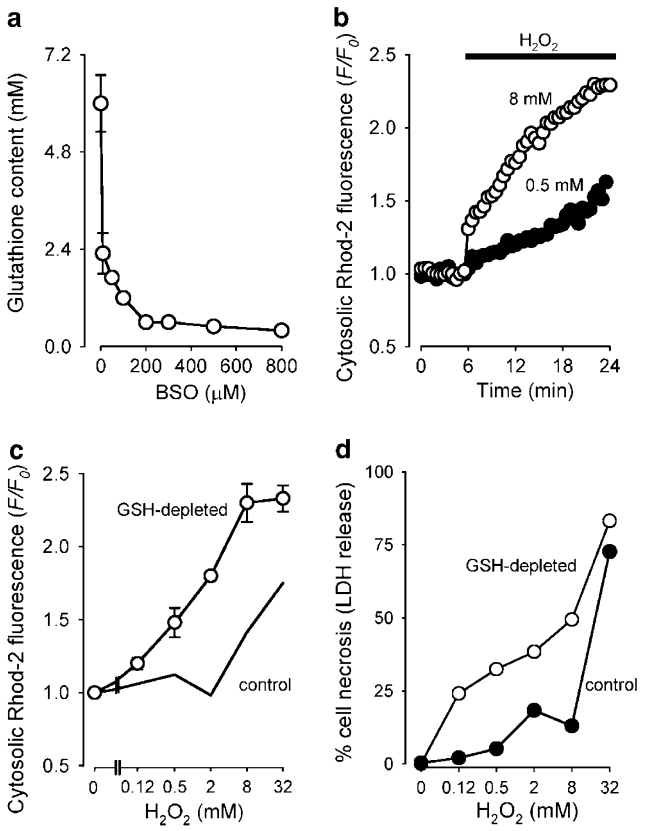

Figure 8 Glutathione depletion increases the sensitivity of H_2O_2-induced calcium increase and necrosis in HeLa cells. (**a**) Cells were exposed for 24 h to increasing concentrations of BSO. Total glutathione concentration was assayed as described in Materials and Methods. (**b**) Cells were first depleted of glutathione with 200 μM BSO for 24 h. Their thapsigargin-sensitive stores were then depleted as in Figure 2. The figure shows the time course of cytosolic calcium signal after exposure to 0.5 and 8 mM H_2O_2. (**c**) Cells depleted as in (**b**) were exposed for 20 min at increasing concentrations of H_2O_2. The dose response obtained in non-GSH-depleted control cells is shown for comparison (taken from Barros *et al.*[22]). Data are means of 8–10 cells. Similar results were obtained in two separate experiments. (**d**) Control cells (closed circles) or glutathione-depleted cells (open circles) were exposed for 3 h to increasing concentrations of peroxide before measurement of necrosis by LDH release as described in Materials and Methods. A similar result was obtained in three other separate assays

and 60$\pm$20%, respectively ($P<0.05$). In contrast, the iron chelator desferroxamine was without effect in either cell type (Figure 9c and d), suggesting that Fenton-derived hydroxyl radicals are not involved in peroxide-induced calcium release. Taken together, these results strongly suggest thiol involvement in peroxide-induced calcium release in epithelial cells and erythrocytes.

Discussion

The main conclusion of this study is that a major component of the early calcium rise associated with oxidative necrosis in epithelial cells originates in the cytosol and not in any of the known intracellular calcium stores. The experiments with mature human erythrocytes confirmed the data from epithelial cells by showing that hydrogen peroxide was able to release calcium with a similar time course and potency in cells devoid of membrane organelles.

In our model of oxidative stress, the early calcium overload was shown to be an important contributing factor to cell necrosis (Figure 1), a result that is consistent with data from renal epithelial cells.[11] In this paper, we did not investigate the events lying downstream of calcium, but in the light of previous data in other systems, several mechanisms of injury may be proposed. In HeLa cells exposed to peroxide, the mitochondria became calcium-loaded, a phenomenon that in neurons mediates glutamate excitotoxicity.[7] The Ca^{2+}-dependent overactivation of poly(ADP-ribose) synthase, which in thymocytes mediates ATP depletion and cell death, is also a likely contributing mechanism, as indicated by the rapid ATP

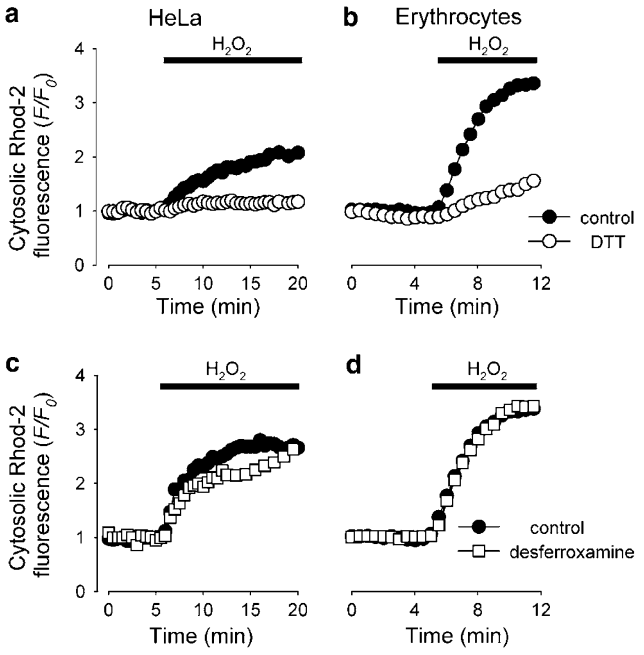

Figure 9 Inhibition of H_2O_2-induced calcium increase by DTT but not by desferroxamine. HeLa cells and erythrocytes were loaded with Rhod-2 as described in Materials and Methods. (**a**,**b**) In both cell types, 5 min pretreatment with 1 mM DTT (open circles) caused a significant inhibition in the rise of intracellular calcium induced by 32 mM peroxide (closed circles). Data are means of 8–10 cells. Similar results were obtained in at least three separate experiments. DTT did not cause detectable changes in cytosolic calcium *per se* (not shown). (**c**,**d**) Pretreatment with 1 mM Fe^{2+} chelator desferroxamine (open squares) for 1 h was without effect on H_2O_2-induced calcium increase (closed circles). Similar results were obtained in at least two separate experiments. Independent assessment of cytosolic calcium with Fluo-3 gave identical results

depletion observed in HeLa cells exposed to peroxide.[22] Another possibility is the activation of calcium-sensitive cation channels, which have been shown to mediate cell death in hepatocytes[14] and astrocytes.[36]

Working with both HeLa cells and MDCK cells, we first ruled out the ER and other thapsigargin-sensitive calcium stores as necessary for the early calcium release. This inference was based on the response elicited by H_2O_2 in cells whose stores had been fully depleted of calcium, a result that is consistent with previous reports on insulin-secreting cells[1] and pancreatic acinar cells,[13] but may appear at variance with data from other cell types in which ER depletion inhibited the calcium increase.[12,37–40] Notwithstanding possible cell idiosyncrasies, the apparent divergence between the two sets of data may relate to the peculiar bell-shaped dose–response curve of both the $InsP_3$ receptor and the ryanodine receptor, which are activated at low doses but inhibited at high doses of oxidants.[41–43] For instance, the two studies on pancreatic cells cited above utilized higher doses of peroxide (i.e. $\geqslant$1 mM) whereas the others used lower doses between 25 nM and 500 μM. It is possible therefore that at low peroxide concentrations, the source of calcium is the ER while at higher concentrations ER release is blocked and the cytosolic source becomes apparent. Such view is further supported by the response of pancreatic acinar cells to the $InsP_3$-receptor antagonist xestospongin C, which was effective at blocking the increase in cytosolic calcium induced by 100 μM peroxide[40] but not by 1 mM peroxide.[13]

Mitochondria were discarded as an early calcium source in HeLa cells based on the observed increase in mitochondrial calcium in response to peroxide, which was much in agreement with the accepted notion that free calcium in these organelles is very low in healthy cells.[30] Moreover, cells treated with the protonophores FCCP and nigericine were still able to release calcium in response to peroxide. The increase was regenerative, similar to the variation in mitochondrial calcium observed in HeLa cells exposed to histamine, thapsigargin or capacitative calcium entry, which show a sharp rise in the rate of mitochondrial calcium uptake over a threshold cytosolic calcium at 400 nM.[17] The threshold is apparently due to the presence of an activatory Ca^{2+} site in the mitochondrial uniporter,[17] and its occurrence here provides indirect evidence for uniporter involvement in peroxide-induced mitochondrial calcium overload. Few minutes after exposure to peroxide, mitochondria started to depolarize, probably by the combined effects of calcium overload and oxidative stress.[8] The late depolarization is expected to release calcium back toward the cytosol, perhaps contributing to the secondary phase of calcium increase often observed in dying cells.

The role of acidic compartments in normal or pathological calcium homeostasis is not well known. These compartments store high μM levels of calcium, and a functional exchange of Ca^{2+} for H^+ occurs at both endosomes[31] and lysosomes.[32] Due to its induction of fast ATP hydrolysis, hydrogen peroxide rapidly decreases cytosolic pH,[25] thus setting the right conditions for accelerated proton/calcium exchange at these organelles. However, abrogation of peroxide-induced cytosolic acidification in HeLa cells using a pH clamp did not significantly affect the calcium rise, showing that proton/calcium exchange is not an important mechanism of oxidative calcium release. As shown by the experiment with Lucifer Yellow, lysosomes remained intact in the first minutes of exposure to peroxide, discarding nonspecific lysosomal membrane disruption as a possible mechanism.

Having ruled out the membranous calcium stores, we sought direct evidence of cytosolic involvement by carrying out experiments in organelle-free human erythrocytes. The response to peroxide in erythrocytes was similar both in extent and dose dependency to that observed in HeLa cells and MDCK cells, therefore demonstrating by an independent approach that no organelles are necessary and that the cytosol (including for this matter the cytosolic face of the plasma membrane) is sufficient to explain the early calcium rise triggered by peroxide.

The identity of the cytosolic molecules that release calcium in response to H_2O_2 is a matter for speculation and further work. Most cytosolic calcium at rest is bound to anionic phospholipids, polyphophoinositides and proteins, with the latter being quantitatively more important.[44] The inhibitory effects of glutathione and DTT suggest that oxidation of sulfhydryl groups in proteins is involved, but a lipid origin in which proteins play a permissive role cannot be ruled out at this stage. Due to its low intrinsic reactivity, H_2O_2 can behave as a quite specific reagent even at high concentrations. For instance, when analyzing the effect of 5 mM peroxide on the

K_m and V_{max} of every enzyme of the glycolytic pathway in P388D1 cells, only GADPH was affected.[45] No such specificity was observed with HOCl, another SH oxidizing reagent, which even at μM concentrations inhibited all 11 enzymes in the pathway.[46] Recently, it has been reported that the exquisite sensitivity of GADPH to peroxide is conferred by a specific selenocysteine located at the catalytic site of the enzyme.[47] Another example of a low-affinity peroxide effect is its inhibition of skeletal muscle Ca^{2+}-activated K^+ channel, which in artificial bilayers showed a $K_i > 10$ mM.[48] A well-known effect of peroxide in combination with transition metals is the production of highly reactive hydroxyl radicals in the Fenton or Haber–Weiss reactions; however, such mechanisms do not seem to be relevant as chelation of transition metals with desferroxamine failed to inhibit the calcium rise. A similar pattern of sensitivity to DTT/insensitivity to desferroxamine has been reported for peroxide-induced calcium rise in human platelets[49] and acinar pancreatic cells.[13]

The novel mechanism of cytosolic calcium release described here in cultured epithelial cells and erythrocytes is likely to be operative in mammalian tissues exposed to pathological oxidative stress. For instance, hydrogen peroxide is found at millimolar concentrations around activated neutrophils[50] and in the skin of vitiligo patients, where it plays a pathogenic role.[51] A recent report on the pathogenic mechanisms of meningitis showed that H_2O_2 released by *Streptoccocus pneumoniae* causes calcium-mediated microglial and neuronal death.[5] This bacteria can produce up to 10 mM H_2O_2 in liquid culture.[52] To the best of our understanding, there are no quantitative measurements of local hydrogen peroxide concentrations in tissues exposed to necrotic ischemia/reperfusion but they are likely to be much higher than the 1 mM required for physiological signaling.[53] At any rate, the results with BSO-treated cells show that the effectiveness of a given peroxide concentration at releasing cytosolic calcium will be largely determined by the local availability of endogenous glutathione. Therefore, cells that in normal conditions are relatively poor in glutathione such as epithelial cells, endothelial cells and heart cells will be more likely to release cytosolic calcium (and die) in response to peroxide. Extreme sensitivity to peroxide would occur when glutathione levels are chronically low such as old age, sickle cell anemia, malnutrition and sepsis. On the contrary, cells enriched in glutathione and related enzyme activities such as preneoplastic cells would be expected to be more refractory to peroxide-induced calcium release.

It is possible that the basic mechanism reported here may also be at work in healthy cells. Oxidant-sensitive fluorescent dyes have shown that normal bulk physiological concentration of H_2O_2 may peak at about 1 mM in vascular smooth muscle.[53] Due to the presence of peroxide-detoxifying enzymes such as catalase and glutathione peroxidase, a steep fall in concentration is to be expected from the immediate vicinity of the production sites toward the bulk cytosol. In healthy cells, hydrogen peroxide is constantly released by the mitochondria, a phenomenon augmented in apoptosis,[54] and it may also be released in large amounts by normal peroxisomes.[55] Mitochondria are tightly associated with ER membranes, determining temporal and spatial microdomains that in the case of calcium ions can reach concentrations many fold higher than those measured in bulk cytosol.[19] By inference, depending on the local balance between production, local degradation and diffusion, an intriguing speculation fuelled by the heightened sensitivity observed in glutathione-depleted cells is that hydrogen peroxide may reach local levels high enough to release cytosolic calcium during normal cell physiology.

Materials and Methods

Chemicals, cell culture and cell viability measurements

HeLa cells, a human epithelial cell line, and MDCK, a canine epithelial cell line, were obtained from the American Tissue Culture Collection. Briefly, cells were grown in DMEM supplemented with 7% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphothericin B at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were passaged at 2- to 5-day intervals and plated on no. 1 glass coverslips in 35 mm Petri dishes. Cells were used between passages 8 and 20 and 2–4 days after plating. Human erythrocytes were obtained from voluntary healthy donors. Histamine, ionomycin, DTT, nigericine, gramicidin, BSO and Lucifer Yellow were purchased from Sigma (St Louis, MO, USA). All other fluorescence dyes, pluronic acid, thapsigargin and BAPTA-AM were purchased from Molecular Probes (Eugene, OR, USA). Desferroxamine was purchased from Ciba-Geigy (Basle, Switzerland). Hydrogen peroxide was from DIFEM-PHARMA (Santiago, Chile) or Merck (Darmstadt, Germany). LDH in cell supernatants was measured[14] using the LDH-P UV kit from Wiener Lab (Rosario, Argentina). Total glutathione was measured by an enzymatic recycling method as described previously.[27] General reagents were purchased from Sigma (St Louis, MO, USA).

Cell fluorescence measurements

Cells were exposed to experimental conditions in Krebs–Ringer–HEPES buffer (KRH; 136 mM NaCl, 10 mM HEPES, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, pH 7.4) supplemented with 25 mM glucose (KRH-glc). In selected experiments, referred to as carried out in nominally calcium-free conditions, extracellular Ca^{2+} was chelated with 10 mM EGTA. All the experiments were carried out at room temperature (22–24°C). Fluorescence was imaged with an LSM 5 Pascal Zeiss confocal microscope by selecting regions of interest (ROIs) under $\times$ 40 or $\times$ 63 objectives. Optical sections were routinely $< 2\,\mu$m thick. Green fluorescent probes Fluo-3, BCECF and Lucifer Yellow were imaged at 488 nm excitation/505–550 nm emission. Red fluorescent probes Calcium Orange 5N, Rhod-2, TMRM and LysoTracker Red were imaged at 543 nm excitation/560–615 nm emission. Routine control experiments were run that confirmed the absence of detectable dye photobleaching. Background noise was measured in segments of the field devoid of cells and found to be not significantly different from the signal recorded in dye-depleted cells (100 μM digitonin or 0.5% Triton X-100). This value was subtracted from cell measurements. In order to compare values from different cells, data were standardized by assigning baseline fluorescence (F_0) a value of 1. Fura-2 was imaged every 1 s with a $\times$ 40 objective using an Olympus microscope fitted with an Optoscan Monochromator System from Cairn (Faversham, UK). Emissions (480–565 nm) were recorded during consecutive 200 ms pulses at 340 and 380 nm excitation and their ratio was calculated after subtraction of signals from a field devoid of cells.

Subcellular Ca^{2+} levels

Calcium was estimated routinely using single-peak dyes, which allowed the use of confocal microscopy to perform simultaneous imaging of a second dye and estimate pH or mitochondrial potential. For confocal microscopy, HeLa and MDCK cells were ester-loaded for 30 min at room temperature with 1 and 5 μM of the high-affinity probes Rhod-2 and Fluo-3, respectively, in KRH-glc containing 0.02% pluronic acid. Erythrocytes were loaded for 1 h at 37°C at the same dye and detergent concentrations. After 30 min de-esterification period, the average fluorescence value of ROIs positioned in the nuclei was registered every 20–30 s. At these time intervals, nuclear and cytosolic calcium levels are in equilibrium in HeLa cells,[56] which was confirmed in our experimental conditions by routinely comparing Fluo-3 data from nuclear and cytoplasmic ROIs. Mitochondrial calcium was estimated by exploiting the preferential loading of positively charged Rhod-2 in these organelles.[17] Peripheral mitochondria were highly mobile, which precluded reliable measurement of individual organelles. Two to five clusters of approximately 10 organelles in the perinuclear region were averaged for every cell. Free calcium in intracellular stores was assessed by loading for 30 min (room temperature) with 1 μM of the low-affinity probe Calcium Orange 5N.[57] Data from individual cells were obtained using perinuclear ROIs. In both HeLa and MDCK cells, Rhod-2 often concentrated in discrete nuclear regions, possibly nucleoli (Figure 5). Incidentally, relative changes in fluorescence from such regions and the nuclear matrix were identical when cells were exposed to histamine or peroxide. Cytosolic calcium was also determined using a ratiometric probe. For this, cells were loaded for 30 min at 37°C with 1 μM Fura-2. Fluorescence data were collected from 8–15 cells per experiment as described above.

Mitochondrial potential

Mitochondrial potential was estimated by loading the cells for up to 2 min with 100 nM TMRM, a concentration that does not affect mitochondrial function.[58] Peripheral mitochondria were highly mobile, which precluded reliable measurement of individual organelles. Two to five clusters of approximately 20 organelles in the perinuclear region were averaged for every cell. TMRM is a cationic dye that crosses lipid membranes with ease but remains in the aqueous phase, distributing in intracellular compartments according to their membrane potential following the Nernst equation.

Cytosolic pH measurement and pH clamping

To estimate cytosolic pH, cells were ester-loaded for 30 min (room temperature) with 1 μM BCECF in KRH-glc. Data were obtained from nuclear ROIs (as describe above). Dye signal was calibrated according to previous reports.[59,60] Briefly, BCECF-loaded cells were incubated in high-potassium buffer (130 mM KCl, 10 mM NaCl, 10 mM HEPES, 1.25 mM $MgSO_4$, 1.25 M $CaCl_2$, 25 mM glucose) adjusted at pH 7.1 for 15 min in the presence of 100 μM nigericin and 10 μM gramicidin. Fluorescence data were then obtained at a range of pH (6.0–7.6), obtained by the addition of KOH to the high-potassium buffer. In selected experiments, the strong cytosolic acidification induced by peroxide was prevented by preincubation with 100 μM nigericin and 10 μM gramicidin in high-potassium buffer (pH clamp). Nigericin is an $H+/K+$ exchanger that clamps the proton gradient across membranes to the reciprocal of the potassium gradient. The latter is in turn forced to 1 by gramicidin, which is a cation ionophore.

Assesment of lysosomal structural integrity

Lysosomes were loaded with Lucifer Yellow by exposing the cells for 30 min to 0.25 mg/ml of the dye in cultured medium, followed by washing and a 4–5 h incubation in culture media.[34] Correct compartmentalization of the dye in lysosomes was ensured by coloading the cells for 10–20 min with 1 nM LysoTracker Red (Figure 6a). As lysosomes are very mobile and high-resolution imaging required a 5 s delay between data collection for the two channels, organelle movement was eliminated by fixation for 10 min with 2% paraformaldehyde prior to imaging. Fixation did not affect the fluorescence of either dye. To assess colocalization, noise was reduced in both channels with a median and a mean filter. Reliable segmentation of Lucifer Yellow/LysoTracker-stained structures was achieved in three steps: (i) enhancing object edges with an isotropic gradient filter, (ii) fixing threshold values in the intensity histograms of these images, and (iii) smoothing binary segments slightly with erosion operators. For time courses, two to four clusters of 10–20 perinuclear lysosomes were averaged for every cell.

Assessment of possible artifacts regarding dye measurements

Several experiments were conducted both with de-esterified dyes and dye-loaded cells to ensure that the high concentrations of H_2O_2 used in some experiments of this paper did not interfere with fluorescent measurements. The results, which are not shown due to space limitations, are as follows. (i) The excitation and emission spectra of Rhod-2, Fluo-3, BCECF and TMRM were unaffected by 32 mM H_2O_2 (LS 55 Perkin-Elmer spectrometer). (ii) The *in vitro* responses of Rhod-2 and Fluo-3 to calcium and the response of BCECF to H^+ were unchanged in the presence of 32 mM H_2O_2. (iii) The increases in Rhod-2 and Fluo-3 signals by peroxide were reversed upon permeabilization of the cells with 2 μM ionomycin in the presence of EGTA. (iv) H_2O_2 exposure did not change the maximum fluorescence of Rhod-2 or Fluo-3 measured in the presence of 2 μM ionomycin in mM calcium. (v) Cell exposure to peroxide for 30 min did not affect the rapid deflection in BCECF intensity induced by a pulse of 25 mM NH_4Cl. In addition, increasing the cytosolic calcium buffering capacity with BAPTA abolished the increase in Rhod-2 fluorescence (Figure 1a), thus discarding possible calcium-independent effects of peroxide on the dye. Similar data were obtained with Fluo-3. An analogous demonstration for cell-loaded BCECF was afforded by the experiment shown in Figure 5 in which pH clamping impeded any detectable change in fluorescence by peroxide. Finally, as reported earlier,[1] the increase in calcium could also be detected with the ratiometric probe Fura-2 (Figure 4b), which demonstrates that possible increases in effective dye concentration do not account for the augmented signal. We conclude that hydrogen peroxide at mM levels does not interfere significantly with calcium or pH measurements using these fluorescent dyes.

Statistical analysis

Data are presented as means $\pm$ standard errors (number of cells). Errors in illustrations were lower than 10% and are omitted for clarity. Differences were assessed using unpaired two-tailed Students's *t*-test, with significance taken at $P<0.05$. Linear regression analysis was performed with SigmaPlot (Jandel corp.).

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