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VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release

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Enhanced formation of reactive oxygen species (ROS), superoxide (O$_2^-$), and hydrogen peroxide (H$_2$O$_2$) may result in either apoptosis or other forms of cell death. Here, we studied the mechanisms underlying activation of the apoptotic machinery by ROS. Exposure of permeabilized HepG2 cells to O$_2^-$ elicited rapid and massive cytochrome c release (CCR), whereas H$_2$O$_2$ failed to induce any release. Both O$_2^-$ and H$_2$O$_2$ promoted activation of the mitochondrial permeability transition pore by Ca$^{2+}$, but Ca$^{2+}$-dependent pore opening was not required for O$_2^-$-induced CCR. Furthermore, O$_2^-$ alone evoked CCR without damage of the inner mitochondrial membrane barrier, as mitochondrial membrane potential was sustained in the presence of extramitochondrial ATP. Strikingly, pretreatment of the cells with drugs or an antibody, which block the voltage-dependent anion channel (VDAC), prevented O$_2^-$-induced CCR. Furthermore, VDAC-reconstituted liposomes permeated cytochrome c after O$_2^-$ exposure, and this release was prevented by VDAC blocker. The proapoptotic protein, Bak, was not detected in HepG2 cells and O$_2^-$-induced CCR did not depend on Bax translocation to mitochondria. O$_2^-$-induced CCR was followed by caspase activation and execution of apoptosis. Thus, O$_2^-$ triggers apoptosis via VDAC-dependent permeabilization of the mitochondrial outer membrane without apparent contribution of proapoptotic Bcl-2 family proteins.

Introduction

The superoxide (O$_2^-$)* free radical has come to play a pivotal role in a wide variety of pathophysiological conditions. In an aerobic system, sequential reduction of molecular oxygen leads to the generation of O$_2^-$, which is rapidly converted to H$_2$O$_2$ by superoxide dismutase (SOD) (Jacobson, 1996). O$_2^-$ can also rapidly react with another free radical, nitric oxide (NO), yielding more toxic reactive oxygen species (ROS), such as peroxynitrite (ONOO$^-$). In physiological systems, for example, ROS play an important role in signaling mechanisms, especially in the TNFα-regulated pathways (Hennet et al., 1993). O$_2^-$ is considered one of the primary ROS generated in cells stimulated by TNFα (Hennet et al., 1993). Furthermore, ROS are known to play a part in proliferation and cell death (Buttke and Sandstrom, 1994; Patel et al., 1996; Fiskum, 2000; Yuan and Yankner, 2000). In several models of apoptosis, increased formation of ROS was described as an early event (Petit et al., 1995; Zamzami et al., 1995a). Most of the ROS have the potential to trigger apoptosis (Buttke and Sandstrom, 1994; Lin et al., 1995; Jacobson, 1996; Hildeman et al., 1999). In addition to enhanced ROS formation, impairment of the cellular antioxidant mechanisms may also lead to apoptosis (Rothstein et al., 1994). Decreased antioxidant profiles were found in epithelial cells that undergo apoptosis (Madesh et al., 1999), and antioxidants have been reported to counter apoptotic cell death (Hockenbery et al., 1993; Zamzami et al., 1995a,b).

Central components of the programmed cell death machinery, which have been conserved throughout evolution, include caspase cascade, Apaf-1 complex formation, and heterodimerization of Bcl-2 family proteins. Cellular organelles, particularly mitochondria, play a crucial role by releasing...
several apoptotic inducing factors, such as cytochrome c (cyto c), Smac/DIABLO, apoptosis-inducing factor (AIF), and caspases from the intermembrane space into the cytosolic environment in response to a variety of apoptotic stimuli (Liu et al., 1996; Adams and Cory, 1998; Green and Reed, 1998; Krajewski et al., 1999; Susin et al., 1999a,b; Du et al., 2000; Verhagen et al., 2000). During apoptosis, translocation of cyto c to the cytosol determines the assembly of a megaprotein complex (Apaf-1). The Apaf-1 complex initially results in activation of caspase-9 followed by the activation of other caspases. Released Smac/DIABLO interacts with the BIR domain of IAPs (inhibitors of apoptosis) and in turn relieves the inhibition of caspases by IAPs (for review see Green, 2000). Ultimately, caspases chop the cellular proteins resulting in programmed cell death.

Mitochondrial dysfunction is one of the prominent features of ROS-mediated cell death. Mitochondrial depolarization, in association with an increased endogenous production of superoxide anion and subsequent damage to the cardiolipins in the inner mitochondrial membrane, was reported as an early event in dexamethasone-induced apoptosis (Petit et al., 1995; Zamzami et al., 1995a,b). A major pathway leading to mitochondrial damage is based on the amplification of mitochondrial and cytosolic superoxide production in a broad spectrum of inflammatory or ischemia-related conditions. However, the exact mechanism by which ROS, and in particular O$_2^-$-mediated apoptosis, establish and utilize CCR remains unclear. Recently, several models have been proposed to elucidate how cyto c and other apoptotic factors are released from mitochondria during apoptosis (for reviews see Green and Reed, 1998; Desagher and Martinou, 2000; Harris and Thompson, 2000; Korsmeyer et al., 2000; Kroemer and Reed, 2000). One paradigm suggests that apoptotic agents trigger mitochondrial permeability transition pore (PTP) opening that results in swelling of the mitochondrial matrix space, causing rupture of the outer mitochondrial membrane (OMM; Marchetti et al., 1996; Zamzami et al., 1996; Kroemer et al., 1998; Bernardi et al., 1999; Crompton, 1999). PTP opening has also been reported to be involved in initiation of the apoptotic machinery without irreversible damage of the mitochondrial membranes (Szalai et al., 1999). ROS and high intramitochondrial Ca$^{2+}$ may act together to trigger PTP opening, and ROS in interaction with Ca$^{2+}$ have been proposed to utilize PTP opening to evoke CCR and subsequent activation of caspases (for review see Zoratti and Szabo, 1995; Ankarcrona et al., 1996; Marzo et al., 1998; Crompton, 1999; Duchen, 2000; Fiskum, 2000; Hajnóczky et al., 2000).

The voltage-dependent anion channel (VDAC, or mitochondrial porin) in the outer membrane and the adenine nucleotide translocator (ANT) in the inner membrane have also been proposed to control release of apoptotic factors without opening of the PTP complex. For example, closure of VDAC was reported to establish a defect in ATP/ADP exchange that results in an inhibition of the F$_1$F$_0$-ATPase and, in turn, an initial hyperpolarization of the inner membrane followed by a loss of the outer membrane integrity and CCR (Vander Heiden et al., 1999).

Other models suggest that release of apoptotic factors occurs via large pores assembled from Bcl-2 family proteins (Bax, Bak) in the mitochondrial outer membrane (for review see Green and Reed, 1998; Desagher and Martinou, 2000; Korsmeyer et al., 2000). Insertion of Bax to the outer membrane and formation of Bax oligomers may provide channels conducting large proteins (Gross et al., 1998; Basanez et al., 1999; Antonsson et al., 2000; Saito et al., 2000). Translocation of tBid from cytosol to the mitochondria have also been proposed to induce an allosteric activation and oligomerization of Bak, forming a pore that allows transport of cyto c (Wei et al., 2000). Bid and Bak may induce cyto c efflux through the OMM without affecting the inner mitochondrial membrane (IMM; von Ahsen et al., 2000). Antiapoptotic Bcl-2 family proteins have been shown to regulate mitochondrial ROS formation (Hockenbery et al., 1993; Kane et al., 1993; Gottlieb et al., 2000) and to inhibit ONOO$^-$-induced CCR from isolated mitochondria distal to ONOO$^-$ formation (Ghafourifar et al., 1999).

Interactions between Bcl-2 family proteins and components of the PTP (ANT, VDAC) have also been implicated in CCR (Marzo et al., 1998; Shimizu et al., 1999). Interaction with Bax has been reported to facilitate CCR via VDAC and this process was inhibited by Bcl-xL in vitro (Shimizu et al., 1999, 2001). Based on these studies, a number of mechanisms may serve as a potential target of ROS during initiation of the mitochondrial phase of apoptosis. Since mitochondrial ATP production is needed for formation of the apoptosome, it is also necessary to determine whether a damage of the IMM causes impairment in oxidative metabolism before CCR.

Here, we aimed to delineate the pathway superoxide radicals utilize to evoke the mitochondrial and cellular phases of apoptosis. We examined the relationship between ROS formation and CCR, the loss of mitochondrial membrane potential ($\Delta$V$_{m}$), caspase activation, and cell death in HepG2 hepatoma cells. We found that O$_2^-$, but not H$_2$O$_2$, induces rapid and massive cyto c release from mitochondria. Although O$_2^-$ and Ca$^{2+}$ acted in a coordinated manner in activation of PTP opening, O$_2^-$-induced CCR was not affected by Ca$^{2+}$ or by PTP blockers. In contrast, VDAC blockers or an anti-VDAC antibody (Ab#25) prevented cyto c release induced by O$_2^-$.

Furthermore, integrity of the IMM was preserved during O$_2^-$-mediated CCR. Bcl-2 family proteins did not appear to be essential for the control of O$_2^-$-mediated CCR. Thus, this study provides evidence for a novel pathway that links O$_2^-$ to VDAC-dependent selective permeabilization of the OMM and CCR, and, ultimately leads to activation of the apoptotic cascade.

Results

O$_2^-$ and H$_2$O$_2$ facilitate Ca$^{2+}$-induced PTP opening, but only O$_2^-$ induces rapid release of cyto c

To examine the effect of O$_2^-$ (generated by xanthine plus xanthine oxidase; X + XO) and H$_2$O$_2$ exposure on PTP, $\Delta$V$_{m}$, and [Ca$^{2+}$], were monitored in permeabilized HepG2 cells incubated in the presence of extramitochondrial ATP supply. Since thapsigargin was added to abrogate endoplasmic reticulum Ca$^{2+}$ uptake, changes in [Ca$^{2+}$], reflected mitochondrial Ca$^{2+}$ uptake and release. We have established a Ca$^{2+}$ pulsing protocol that evoked modest depolarization and rapidly decaying [Ca$^{2+}$], transients in naive cells,
whereas large and extended depolarization and \([Ca^{2+}]_c\) elevation in cells treated with agents that promote the gating of PTP by \(Ca^{2+}\) (Szalai et al., 1999). As shown in Fig. 1 A, upon addition of \(X + XO\) the mitochondrial depolarization evoked by \(Ca^{2+}\) pulses was augmented and prolonged. Simultaneously, mitochondria in the \(X + XO\)–pretreated cells lost their capacity to keep sequestered the exogenously added \(Ca^{2+}\). Enhanced depolarization and impaired \(Ca^{2+}\) uptake exhibited by \(X + XO\)–treated cells reflected opening of PTP as it was inhibited by cyclosporin A (CsA; see Fig. 3 A). The mitochondrial depolarization and \([Ca^{2+}]_c\) rise in \(X + XO\)–treated cells were also attenuated and shortened by pretreatment with an \(O_2^-\) scavenger, superoxide dismutase mimic (Mn[III]tetakis [4-benzoic acid] porphyrin, MnTBAP, 20 \(\mu\)M for 5 min; Fig. 1 A), suggesting that \(O_2^-\) formation promoted the activation of PTP by \(Ca^{2+}\).

Similar to \(O_2^-\), \(H_2O_2\) potentiated the \(Ca^{2+}\) pulsing–induced depolarization and \([Ca^{2+}]_c\) elevation (Fig. 1 B). These effects of \(H_2O_2\) were inhibited by an \(H_2O_2\) scavenger, catalase (Fig. 1 B) and by CsA (unpublished data; \(n = 2\)). This finding suggests that \(H_2O_2\) also promoted \(Ca^{2+}\)–dependent PTP opening.

At the end of fluorometric measurements, samples were centrifuged and the cytosol was separated from the membrane fraction. In the control, most of the cytochrome c was detected only in the mitochondrial pellet (Fig. 1 C). In \(X + XO\)–pretreated cells (\(X + XO\) for 375 s and \(Ca^{2+}\) for 300 s), less cytochrome c was in the pellet and a large amount was observed in the cytosol. Importantly, in the presence of MnTBAP, \(X + XO\) did not cause cytochrome c redistribution from mitochondria to cytosol (Fig. 1 C). Similarly, pretreatment with SOD (500 U/ml) also prevented the CCR from mitochondria (unpublished data, \(n = 2\)). In contrast to \(O_2^-\), \(H_2O_2\) \((0.9–90 \text{ mM})\) did not elicit cytochrome c rise in the cytosol (Fig. 1, C and D). \(O_2^-\) rapidly reacts with NO, yielding ONOO\(^-\), which has also been shown to evoke CCR and apoptosis (Lin et al., 1995; Ghafourifar et al., 1999). To evaluate the possibility of involvement of ONOO\(^-\) in \(X + XO\)–dependent CCR, per-
meabilized cells were pretreated with various concentrations of an ONOO− scavenger, ebselen, and then exposed to O2−. O2−-induced CCR was not inhibited in the concurrent presence of ONOO− scavenger (Fig. 1 E). Together, these results demonstrate that O2− and H2O2 promote Ca2+-dependent PTP opening, whereas O2−, but not H2O2 or the O2− product, ONOO−, selectively induced CCR.

To quantitate O2−-dependent CCR, various amounts of cytosolic and membrane proteins were resolved on SDS-PAGE. The amount of CCR was compared with known quantities of cyto c standard. As shown in Fig. 1 F, the vast majority of mitochondrial cyto c was released to the cytosol in permeabilized cells exposed to X + XO (5 mU/ml) for 375 s. Thus, the loss of cyto c from mitochondria in response to O2− treatment is rapid and almost complete.

**O2−-induced CCR is independent of PTP opening**

To investigate the mechanism by which O2− induces CCR, simultaneous measurements of ΔΨm and [Ca2+]i were performed at various concentrations of X + XO in the absence and presence Ca2+ pulses (Fig. 2). In the absence of Ca2+, mitochondrial depolarization was not detectable during 375 s exposure to O2−-generating system (Fig. 2 A). When Ca2+ was added to the permeabilized cells, depolarization was seen in a dose-dependent manner (Fig. 2 B). To determine cyto c distribution, cytosol and membrane fractions were separated as described above. Interestingly, O2− was able to induce CCR in a dose-dependent manner, even in the absence of Ca2+ addition (Fig. 2 C). CCR was observed at ≈4 mU XO and it was almost complete. Addition of Ca2+ did not affect the sensitivity of CCR to X + XO. Collectively, these data show that Ca2+ is not required for O2−-dependent CCR.

To determine whether opening of PTP was involved in O2−-induced CCR, permeabilized cells were treated with inhibitors of the inner membrane components of the PTP, CsA (1 μM), or bongkrekic acid (BA; 10 μM) before the addition of X + XO and Ca2+ pulses (Fig. 3). As shown in Fig. 3 A, CsA that binds to cyclophilin D in the mitochondrial matrix prevented the Ca2+-induced depolarization and sustained [Ca2+]i rise in X + XO-treated permeabilized cells. By contrast, CsA failed to affect the CCR evoked by X + XO both in the presence or absence of Ca2+ (Fig. 3 B). Furthermore, BA, a blocker of the adenine nucleotide translocator that spans the IMM, was also ineffective to attenuate O2−-induced CCR (Fig. 3 C). ADP supports the binding and inhibitory effect of BA, but BA also failed to inhibit O2−-induced CCR when the ATP regenerating system was omitted from the medium to prevent extramitochondrial phosphorylation of ADP (Fig. 3 C). Together, these results show that opening of PTP is not required for O2−-induced CCR.

**Preservation of ΔΨm requires extramitochondrial ATP supply in cells exposed to O2−**

Following release of most of the mitochondrial cyto c (Fig. 2), oxidative metabolism is likely to be impaired. Thus, ΔΨm could be maintained in the presence of X + XO in expense of the extramitochondrial ATP supply (Fig. 2 A). To test whether reversed operation of the F0F1-ATPase allowed extrusion of H+ in mitochondria exposed to O2−, we added an inhibitor of the F0F1-ATPase, oligomycin, and monitored the effect of X + XO on ΔΨm. Under these conditions X + XO caused rapid and complete depolarization (Fig. 4 A). CsA did not affect the X + XO-induced depolarization in oligomycin-pretreated cells (Fig. 4 A). Notably, pretreatment with oligomycin did not change the amount of CCR induced by O2− (unpublished data).

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**Figure 2.** Ca2+-induced mitochondrial depolarization is not essential for O2−-induced CCR. Permeabilized cells were treated with X (0.1 mM) and different concentrations of XO (0.1, 1, 2, 3, 4, 5, 10, or 20 mM) before addition of Ca2+ pulses (30 μM each) or solvent. (A and B) ΔΨm measured simultaneously with [Ca2+]i. (C and D) cyto c in the cytosol and membrane fractions. Immunoblotting of actin in the cytosolic fraction was used to evaluate whether O2− changed the distribution of nonmitochondrial proteins.
O$_2$-induced CCR precedes the $\Delta\Psi_m$ loss

To clarify the temporal relation between CCR and $\Delta\Psi_m$ loss, the time-course of CCR and depolarization were studied after exposure to X + XO in oligomycin-pretreated permeabilized cells. As shown in Fig. 5 A, CCR in the cytosol was apparent at 125 s and maximum release was observed at 225 s after X + XO treatment. CCR was associated with a decrease in $\Delta\Psi_m$ that started >200 s after O$_2$-addition (Fig. 4 A). Our results suggest that the loss of $\Delta\Psi_m$ follows the O$_2$-induced rapid release of cyto c instantaneously.

If depolarization was due to cyto c depletion–induced impairment of $\Delta\Psi_m$ generation by the oxidative pathway, supply of exogenous cyto c to the bulk cytosolic buffer may sustain $\Delta\Psi_m$ in oligomycin-treated cells (Waterhouse et al., 2001). As shown in Fig. 4 B, exogenously added cyto c (2, 5, and 10 μM) attenuated O$_2$-induced $\Delta\Psi_m$ loss in a dose-dependent fashion. These results show that after O$_2$-induced CCR, mitochondria can maintain $\Delta\Psi_m$ only if ATP was provided by an extramitochondrial source or the decrease in the intramitochondrial concentration of cyto c was compensated by added cyto c. Also, these results suggest that the IMM was intact even after O$_2$-induced CCR.

Inhibitors of VDAC abrogate O$_2$-induced CCR

Recent studies have reported that Bax, a proapoptotic Bcl-2 family protein may interact with VDAC to trigger CCR. It is also to note that the antiapoptotic protein, Bcl-xL prevents the O$_2$-induced CCR from mitochondria through the VDAC–Bax complex (Shimizu et al., 1999). The OMM contains a large amount of VDAC, and this protein channel can act as a track for the transport of substances in and out of mitochondria (Colombini et al., 1996). Thus, we next investigated the effect of VDAC blockers, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (Shafir et al., 1998) and Konig’s polyanion (KP), on the O$_2$-induced CCR. Pretreatment with DIDS (50 μM) for 1 min before the addition of X + XO completely inhibited O$_2$-induced CCR (Fig. 5 A). Permeabilized cells were also pretreated with different concentrations of KP for 100 s before exposure to X + XO. Treatment with KP also inhibited CCR induced by O$_2$ (Fig. 5 B). At higher concentrations, the KP caused release of a fraction of cyto c by itself. These results indicate that VDAC is involved in the O$_2$-induced CCR.

Recently, Shimizu et al. (2001) described an anti-VDAC antibody (Ab#25) that inhibits VDAC activity. We used this antibody to further verify the role of VDAC in CCR.

Figure 3. PTP blockers fail to inhibit O$_2$-induced CCR. Permeabilized cells were pretreated with CsA (1 μM) or BA (10 μM) before the addition of X + XO. (A) Mitochondrial depolarization and [Ca$^{2+}$]$_c$ rise evoked by Ca$^{2+}$ pulses added after the pretreatment with X + XO or CsA + X + XO. (B and C) Released cyto c in the cytosol was visualized by Western blotting. Cytosol samples were prepared after the measurement of $\Delta\Psi_m$ but FCCP was not added. Permeabilized cells were pretreated with BA (10 μM) in the presence or absence of ATP-regenerating system before the X + XO treatment. Because ADP supports the binding and inhibitory effect of BA, to prevent extramitochondrial phosphorylation of ADP, ATP-regenerating system was omitted from the medium in some experiments.

Figure 4. O$_2$-induced depletion of mitochondrial cyto c yields mitochondrial depolarization in the absence of extramitochondrial ATP supply. (A) Effect of O$_2$ on $\Delta\Psi_m$ was monitored in permeabilized cells pretreated with oligomycin (2.5 μg/ml), an inhibitor of the FoF1-ATPase before the addition of X + XO or CsA + X + XO. (B) Rescue of mitochondria from O$_2$-induced depolarization by exogenously added cyto c. Permeabilized cells were preincubated with cyto c (2, 5, or 10 μM) and oligomycin (2.5 μg/ml) before exposure to X + XO.
and $\Delta \Psi_m$ loss of permeabilized cells treated with X + XO. In permeabilized cells pretreated with oligomycin, X + XO showed massive amount of CCR and large $\Delta \Psi_m$ loss. However, cells pretreated with Ab#25 (0.56 $\mu$g/µl) for 5 min prevented the CCR (Fig. 5 D) and the $\Delta \Psi_m$ loss (Fig. 5 C).

**Involvement of proapoptotic Bcl-2 family proteins is not necessary for $O_2^-$-induced CCR**

The interaction of Bcl-2 family proteins in the mitochondrial membranes can control the permeability to cyto c. During apoptosis, translocation of Bax from cytosol to mitochondria facilitates the release of cyto c through the OMM (Eskes et al., 1998; Jurgensmeier et al., 1998; Marzo et al., 1998; Narita et al., 1998; Pastorino et al., 1998). Bax has also been reported to interact with VDAC to release cyto c (Shimizu et al., 1999). However, in our study, cytosolic Bax did not translocate to the mitochondrial membrane during O$_2^-$ exposure (Fig. 5 E). By contrast, cells exposed to a proapoptotic agent, staurosporine (1 $\mu$M) for 3 h showed significant amount of mitochondrial anchored Bax. Furthermore, washout of the cytosol after cell permeabilization did not prevent O$_2^-$-mediated CCR (unpublished data; n = 3). These data indicate that O$_2^-$-induced CCR through VDAC does not require translocation of Bax to the mitochondria.

Another proapoptotic Bcl-2 family protein, Bak, may substitute for Bax in the CCR (Wei et al., 2001). However, no Bak was detected in the lysate of HepG2 cells using a monoclonal antibody (anti-human Bak, clone TC102; Oncogene Research Products) that recognized Bak in the lysate of H9c2 cardiac myocytes (unpublished data). Thus, Bak does not appear to be necessary for the O$_2^-$-induced CCR.

**Coupling of caspase activation to O$_2^-$-mediated CCR**

Mitochondrial permeabilization and CCR into the cytosol are essential events for caspase activation (Liu et al., 1996). We next examined whether the O$_2^-$-induced release of cyto c and, presumably, other mitochondrial factors, is associated with caspase activation. After a short exposure to O$_2^-$, cleavage of pro-caspase-3 was detected, suggesting caspase-3 activation (Fig. 6 A). The VDAC blocker DIDS completely blocked the O$_2^-$-mediated pro-caspase-3 cleavage, whereas CsA or BA did not prevent O$_2^-$-mediated caspase activation (Fig. 6 A). One may speculate that the disappearance of pro-caspase-3 was due to its degradation by a necrosis-stimulating protease. To evaluate formation of the active caspase-3 enzyme, we also performed measurements of the cleavage product of a fluorogenic caspase substrate, DEVD-AMC (Fig. 6 B). In extracts of X + XO–treated permeabilized cells, the cleavage of DEVD-AMC was augmented. This effect was abolished by DIDS, whereas CsA did not attenuate the X + XO–stimulated DEVD-AMC cleavage (Fig. 6 B). We also observed that a caspase inhibitor, Ac-DEVD-CHO (50 $\mu$M), did not affect CCR, but completely blocked caspase-3 activation (unpublished data; n = 2). Thus, O$_2^-$ resulted in caspase activation which was closely coupled to VDAC-dependent CCR.

**O$_2^-$ evokes VDAC-dependent CCR in intact cells**

To demonstrate whether O$_2^-$ can also trigger CCR in intact cells, HepG2 cells were treated with X + XO for various time periods. The time-course of the exposure to O$_2^-$ during treatment with X + XO was evaluated fluorometrically, using a ROS tracer, 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes). A substantial increase in ROS was noticed after 5 min and a plateau was reached after 60 min treatment with X + XO (n = 2). To study the cyto c distribution, after treatment with X + XO, cells were harvested, permeabilized and the cytosol was separated from the membrane fraction by centrifugation. Naive cells and cells treated with X + XO for a short duration (15 or 30 min) did not have cyto c in the cytosol (Fig. 7 A). Sometimes, we de-
localized in mitochondria (Fig. 7 C). Cyto c–GFP appeared in the cytosol and in the nuclear matrix after 1 h or longer treatment with X + XO (Fig. 7 C). Cell to cell differences in the lag time of cyto c–GFP redistribution were observed, but once the release started it rapidly involved every mitochondrion. At 3 h of exposure to X + XO, cyto c–GFP distribution was homogeneous in most of the cells (Fig. 7 C). The O$_2^-$–induced release of cyto c–GFP was quantitated based on the fluorescence appearing in the nuclear region of the cells (Fig. 7 D). The onset of cyto c–GFP release was similar to that of CCR, whereas the maximal release of cyto c–GFP was obtained 5 h after addition of X + XO (Fig. 7 D). Pretreatment with CsA (5 μM) did not inhibit cyto c–GFP release from mitochondria after O$_2^-$ exposure. However, DIDS prevented the release of cyto c (Fig. 7 D). Collectively, these studies demonstrated that increased formation of O$_2^-$ is capable of evoking CCR in intact cells and further supported the role of VDAC in this process.

Selective permeabilization of the OMM by truncated Bid has been described to initiate CCR in the absence of major damage of the mitochondrial structure (von Ahsen et al., 2000). Using transmission electron microscopy, we evaluated the morphology of the mitochondria in intact cells incubated in the absence and presence of X + XO for 1 h (Fig. 7 E). Consistent with previous studies in HepG2 cells (e.g., Lewis et al., 1996), the majority of the mitochondria appeared in close association with the endoplasmic reticulum, was oval shaped, and was packed with cristae (left). Furthermore, 1 h of treatment with X + XO did not cause a major change in the mitochondrial structure (right). Thus, the onset of the CCR was not preceded by X + XO–induced large amplitude swelling of the mitochondria.

**O$_2^-$ evokes cyto c efflux from liposomes reconstituted with VDAC**

To further explore the role of VDAC in O$_2^-$–mediated apoptosis, we examined the effect of O$_2^-$ on the permeability of VDAC purified (confirmed by Coomassie staining and immunoblotting; Fig. 8 A) from liver mitochondria and incorporated into liposomes. Plain liposomes and VDAC liposomes loaded with cyto c–FITC were exposed to O$_2^-$ for 7 min, and the distribution of cyto c–FITC was visualized using confocal microscopy (Fig. 8 B). Plain, cyto c–FITC–loaded liposomes displayed only a small change in fluorescence distribution in response to X + XO (top images; extraliposomal fluorescence: 6.4 ± 0.6 and 11.4 ± 0.3 arbitrary units before and after X + XO addition, respectively; n = 3). By contrast, VDAC reconstituted liposomes allowed for a large increase of extraliposomal fluorescence in response to O$_2^-$ exposure (middle images; 6.9 ± 0.2 and 33.3 ± 0.7 arbitrary units before and after X + XO addition, respectively; n = 3). Involvement of VDAC in the O$_2^-$–induced CCR was further tested using DIDS. Pretreatment with DIDS (100 μM) inhibited the X + XO–induced increase in fluorescence between VDAC liposomes (bottom images; 7.6 ± 0.3 and 15.5 ± 0.5 arbitrary units before and after X + XO addition, respectively; n = 3).

To quantify cyto c–FITC release from the liposomes, we also centrifuged liposome suspensions and measured the fluorescence in the supernatant and in the pellet using a flu-
orimeter. As shown in Fig. 8 C, the fluorescence in the supernatant was small when liposomes were prepared without cyto c–FITC loading and reconstitution ofVDAC, exposure to X + XO or DIDS did not evoke any changes (bars: 1, 3, 5, 7, and 9). When cyto c–FITC–loaded plain liposomes were exposed to X + XO, the fluorescence in the supernatant showed a twofold increase, similar to the data obtained with confocal imaging (bar 4 vs. 2). This increase was due to a direct interaction between the fluorophore and O$_2^-$, because a comparable increase was obtained when FITC was incubated with X + XO (unpublished data). However, the supernatant prepared from suspensions of VDAC liposomes exhibited eightfold increase after exposure to X + XO (bar 8 vs. 6). Comparing the plain and VDAC liposomes, incubation with X + XO caused a much larger increase of the fluorescence in the supernatant (bar 8 vs. 4) and this was accompanied with a much larger decrease of the fluorescence in the pellet prepared from VDAC liposomes (unpublished data, n = 2). In addition, X + XO–induced redistribution of the fluorescence was significantly attenuated by pretreatment with DIDS (bar 10 vs. 8). Importantly, Bax was not detected in purified VDAC or in VDAC liposomes, providing further evidence that the effect of O$_2^-$ on VDAC was not mediated by Bax (Fig. 8 A). Together, these studies show that reconstitution of VDAC in liposomes allows for a O$_2^-$–induced increase in cyto c permeability. The lack of O$_2^-$–dependent efflux of cyto c–FITC from plain liposomes suggests that O$_2^-$ does not rupture the lipid domains of the mitochondrial outer membrane. These results further confirmed that O$_2^-$ modulates opening of VDAC and facilitates CCR. The fact that VDAC blockers and anti-VDAC antibody prevent the CCR in the HepG2 cells and that PTP blockers did not prevent O$_2^-$–induced CCR suggested that the ability of O$_2^-$ to interact with VDAC is sufficient to induce CCR from mitochondria.

Execution of apoptosis in cells exposed to O$_2^-$

We have demonstrated CCR and caspase activation in cells exposed to O$_2^-$ . To determine whether CCR and caspase activation are followed by ordered execution of the apoptotic program, phosphatidylserine (PS) exposure was evaluated by annexin V staining, and membrane permeability was studied by labeling with propidium iodide (Fig. 9). In X + XO–treated cells, imaging of ΔΨ$_m$ simultaneously with Alexa 488 annexin showed an early mitochondrial depolarization (Fig. 9 A; decrease in red signal at 1 h). Presumably, extramitochondrial ATP production is relatively small in HepG2 cells and therefore mitochondrial depolarization was closely coupled to cyto c release. After 1 h of X + XO treatment, very few cells exhibited annexin labeling of the plasma membrane, whereas at 3 h most of the cells were positive (green signal). Annexin staining of the cell could occur due to permeabilization of the plasma membrane; however, in cyto c–GFP–expressing cells, the cyto c–GFP released from mitochondria to the cytosol did not leave the cells, suggesting that the plasma membrane barrier was preserved (Fig. 9 B). Also, propidium iodide was initially excluded from the PS-positive cells (Fig. 9 C, see below). Treatment with DIDS (100 μM) attenuated the O$_2^-$–induced ΔΨ$_m$ loss and annexin staining (unpublished data). These observations suggest that execution of the apoptotic program was initiated by O$_2^-$ through a VDAC-dependent signaling pathway.
Apoptotic cells can be differentiated from necrotic cells based on the fact that apoptotic cells become permeable to propidium iodide (PI) relatively late. To evaluate the contribution of apoptosis and necrosis to cell death, after exposure to O$_2^{-}$, we quantitated apoptosis and necrosis by annexin V and PI staining, suggesting the onset of late stage apoptosis (Fig. 9 C). Thus, CCR and mitochondrial depolarization, which occurred at 1–2 h X + XO treatment (Figs. 7 A and 9 A), were followed by phosphatidylserine exposure and permeabilization of the plasma membrane was further delayed. This order of events suggests that the cells went through apoptotic cell death. This conclusion was further supported by the fragmentation of DNA in the cells exposed to O$_2^{-}$ (Fig. 9 D).

**Discussion**

This study describes a novel mechanism underlying activation of CCR by O$_2^{-}$, but not by H$_2$O$_2$ or ONOO$^-$. We showed that O$_2^{-}$-induced CCR takes place quickly and involves most of the cyto c stored in the mitochondria. Although ROS interact with Ca$^{2+}$ in gating of PTP, we have shown that the ROS-induced CCR pathway is independent of the inner membrane components of the pore. Our data suggest that the release of cyto c by O$_2^{-}$ is mainly due to VDAC-dependent selective permeabilization of the mitochondrial outer membrane. Ultimately, these alterations lead to activation of caspases and execution of apoptosis.

We examined the molecular machinery used by ROS to trigger the mitochondrial phase of apoptosis. The first important observation was that O$_2^{-}$ generated by the xanthine/xanthine oxidase system caused rapid release of cyto c from mitochondria to the cytosol in intact as well as permeabilized HepG2 cells. Similar to cyto c, Smac/DIABLO was also rapidly released by O$_2^{-}$ (unpublished data). O$_2^{-}$-induced release of cyto c and Smac/DIABLO may have a widespread significance in apoptosis, since in a number of pathologic conditions, such as ischemia/reperfusion injury, drug insults, and inflammatory responses, large amounts of O$_2^{-}$ are produced by xanthine oxidase–mediated catabolism of purine nucleotides or by increased electron transport chain activity or by activation of NADPH oxidase particularly in neutrophils (Granger et al., 1986; Babior, 1999). Furthermore, increased formation of O$_2^{-}$ was described as an early event in several apoptotic paradigms (Petit et al., 1995; Zamzami et al., 1995a).

In the cells, O$_2^{-}$ can be converted to other ROS (H$_2$O$_2$, ONOO$^-$), which have been reported to evoke CCR from isolated mitochondria (Yang and Cortopassi, 1998; Ghafoorifar et al., 1999). However, in our studies, H$_2$O$_2$ (90 mM) did not establish rapid CCR and a ONOO$^-$ scavenger, ebselen did not attenuate the O$_2^{-}$-induced CCR. Thus, we conclude that O$_2^{-}$-triggered CCR by itself.

A principal mechanism of the CCR could involve ROS-induced promotion of Ca$^{2+}$-dependent PTP opening. Permanent PTP opening causes CCR via matrix swelling and rupture of the mitochondrial membranes (for review see...
The mitochondria were provided with exogenous cyto-
 annexin V Alexa 488 and TMRE. (B) Distribution of cyto-
 membrane integrity, and DNA fragmentation in cells exposed to
 O2\(^{-}\). Cells were treated with X + XO (0.1 mM plus 20 mU/ml) for
 various time periods in the absence or presence of DIDS (100 μM).
 (A) \(\Delta\Psi_m\) and PS exposure were evaluated with confocal imaging
 of annexin V Alexa 488 and TMRE. (B) Distribution of cyto c-GFP and
 annexin staining were visualized. (C) PS exposure and loss of
 plasma membrane integrity were evaluated with imaging of annexin
 V Alexa 488 and propidium iodide. (D) DNA fragmentation analysis
 was performed with cells incubated in the presence or absence of
 X + XO for 12 h.

Figure 9. Execution of apoptosis in HepG2 cells exposed to O2\(^{-}\).
Mitochondrial depolarization, PS exposure, disruption of plasma
 membrane integrity, and DNA fragmentation in cells exposed to
 O2\(^{-}\). Cells were treated with X + XO (0.1 mM plus 20 mU/ml) for
 various time periods in the absence or presence of DIDS (100 μM).
 (A) \(\Delta\Psi_m\) and PS exposure were evaluated with confocal imaging
 of annexin V Alexa 488 and TMRE. (B) Distribution of cyto c-GFP and
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 V Alexa 488 and propidium iodide. (D) DNA fragmentation analysis
 was performed with cells incubated in the presence or absence of
 X + XO for 12 h.

Kroemer et al., 1998; Bernardi et al., 1999; Crompton,
 1999; Desagher and Martinou, 2000), but reversible PTP
 opening was also shown to set up partial CCR without irre-
 versible damage of mitochondrial function (Szalai et al.,
 1999). We also observed that Ca\(^{2+}\)-induced PTP opening
 was amplified in permeabilized cells exposed to O2\(^{-}\) or
 H2O2, and simultaneous presence of either O2\(^{-}\) scavengers
 SOD or SOD mimic, MnTBAP, or H2O2 scavenger catalase
 attenuated this effect. However, O2\(^{-}\)-induced CCR was in-
 sensitive to inhibitors of PTP (CsA, BA) and was also
 observed in the absence of Ca\(^{2+}\). Thus, PTP opening is not
 essential for O2\(^{-}\)-induced CCR. In addition, we demon-
 strated that O2\(^{-}\)-induced CCR does not yield \(\Delta\Psi_m\) loss if
 the mitochondria were provided with exogenous cyto c or
 ATP. These data suggest that integrity of the mitochondrial
 inner membrane and matrix space was preserved during
 O2\(^{-}\)-induced CCR. It is important to note that in intact
 cells, mitochondrial depolarization was closely coupled to
 CCR, providing evidence that CCR caused impairment in
 the function of the electron transport chain and extramito-
ochondrial ATP production could not maintain mitochon-
drial proton extrusion. Thus, O2\(^{-}\)-induced CCR is medi-
 ated by selective permeabilization of the OMM and the
 O2\(^{-}\)-induced mitochondrial depolarization was secondary to
 the loss of mitochondrial cyto c.

In regard to the changes in mitochondrial function, the
 origin of O2\(^{-}\) is likely to be of great significance. O2\(^{-}\)
 produced by the respiratory chain has been reported to cause
 cardiolipid destruction in the IMM and dissipation of the
 \(\Delta\Psi_m\) (Zamzami et al., 1995a). However, based on our data,
 O2\(^{-}\) produced by X + XO in the cytosol or in the extracel-
 lular medium elicits selective permeabilization of the OMM
 and the damage of the IMM may occur after impairment of
 the OMM barrier. Presumably, slow permeation of O2\(^{-}\)
 through the OMM and the ROS scavengers in the mito-
 chondria may attenuate exposure of the IMM to O2\(^{-}\) pro-
 duced outside the mitochondria. Protection of the IMM
 may be particularly effective when a small amount of O2\(^{-}\)
 is produced, whereas a robust increase in extramitochondrial
 O2\(^{-}\) may cause an early damage of both the OMM and
 IMM and, in turn, necrosis (Higuchi et al., 1998). Along
 this line, HepG2 cells are expected to exhibit necrosis when
 exposed to a high dose of O2\(^{-}\). Interestingly, 100 mU XO
 plus 0.1 mM X was not sufficient to cause necrosis in all
 HepG2 cells, since DNA fragmentation was also noticed in
 this condition. Notably, cultured cells grown in 20% oxygen
 are essentially preadapted to conditions of oxidative stress
 (e.g., Davies, 1999).

Selective permeabilization of mitochondrial outer mem-
 brane may be established by pores formed by Bcl-2 family
 proteins (for review see Green and Reed, 1998; Desagher
 and Martinou, 2000; Korsmeyer et al., 2000) or by pores
 produced by the interaction of VDAC and Bax (Shimizu et
 al., 1999, 2001). Here, we showed that O2\(^{-}\)-induced CCR was
 inhibited by VDAC blockers (KP, DIDS) as well as by an
 antibody that interferes with the function of VDAC
 (#25; Shimizu et al., 2001). Furthermore, liposomes reconsti-
 tuted with purified VDAC displayed O2\(^{-}\)-induced release of
 FITC-cyto c. Together, these data demonstrate that
 VDAC plays a central role in O2\(^{-}\)-induced CCR. In the
 studies of Shimizu et al. (1999), permeation of cyto c by
 VDAC liposomes required addition of Bax. By contrast, in
 our studies, O2\(^{-}\)-induced release of cyto c from the lipo-
 somes did not depend on the presence of Bax. The present
 study also showed that in normal HepG2 cells, the mono-
 meric Bax form was present in the cytosol and translocated
 into the mitochondrial membrane upon staurosporine treat-
 ment. In contrast, cells exposed to the O2\(^{-}\)-generating sys-
 tem did not show any translocation of Bax into mitochon-
 dria, even at high concentrations of X + XO, but almost
 complete release of cyto c was still observed. On the same
 line, washout of the cytosol did not attenuate O2\(^{-}\)-induced
 CCR in the permeabilized cell experiments. Collectively,
 these data suggest that O2\(^{-}\) directly targets VDAC or a fac-
 tor that is tightly coupled to VDAC in the process that es-
 tablished CCR and Bax does not mediate the effect of O2\(^{-}\)
 on VDAC.

In the cytosol, released cyto c can bind to Apaf-1 and up-
 stream caspases to form a complex, where they are cleaved
 into their active forms (Zou et al., 1997). Activated caspase-9
can process inactive procaspase-3 into active caspase-3 and thereby triggers intracellular disintegration (Li et al., 1997). Our studies demonstrated that O$_2^-$ triggered caspase-3 activation and similar to O$_2^-$-induced CCR, caspase activation was not inhibited by PTP blockers (CsA or BA), whereas it was prevented by DIDS. In intact cells, O$_2^-$-induced CCR was followed by mitochondrial depolarization, PS exposure and finally the cells become propidium iodide positive. This sequence of events suggests that O$_2^-$-induced execution of the apoptotic death program. The apoptotic process exhibits rapid progression and takes place in most of the cells exposed to O$_2^-$, suggesting that O$_2^-$ may play a fundamental role in apoptosis caused by enhanced ROS production.

Materials and methods

Cell culture and transfections

HepG2, human hepatocellular carcinoma cells were grown in minimum essential medium (MEM; GIBCO-BRL) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and 2 mM glutamine at 37°C in 5% CO$_2$. For imaging experiments, cells were plated onto poly-d-lysine-treated glass coverslips (2–3 × 10$^5$cm$^2$) and were grown for 72 h. For cell suspension studies, cells were cultured for 0–5 d in 75 cm$^2$ flasks, and were harvested by brief treatment with trypsin-EDTA. For transient transfections, cells were transfected using plasmid DNA (cyto-c-GFP; Heiskanen et al., 1999) keeping total DNA at 2 μg/ml and cationic lipid Lipofecta-AMINE 2000 according to the manufacturer’s instructions. After 5 h, the cells were placed in a normal growth medium. Imaging was performed 48 h after transfection.

Measurement of ΔΨm and [Ca$^{2+}$]i in suspension of permeabilized cells

Cells were washed in ice cold Ca$^{2+}$-free extracellular buffer containing 120 mM NaCl, 5 mM KCl, 1 mM KH$_2$PO$_4$, 0.2 mM MgCl$_2$, 0.1 mM EGTA, 20 mM Hepes-NaOH, pH 7.4. An equal amount of cells (9–10 × 10$^6$ cells, 7 mg/ml protein) were resuspended and permeabilized with 40 μg/ml digitonin in 1.8 ml of intracellular medium (ICM) composed of 120 mM KCl, 10 mM NaCl, 1 mM KH$_2$PO$_4$, 20 mM Hepes-Tris, pH 7.2, supplemented with 1 μg/ml of each of antipain, leupeptin, and pepstatin. ICM was passed through a Chelex column before addition of protease inhibitors to lower the ambient Ca$^{2+}$. All the measurements were performed in the presence of 2 mM MgATP, 2 mM succinate, and ATP-regenerating system composed of 5 mM phosphate and 5 U/ml creatine kinase.

For the simultaneous measurements of ΔΨm and mitochondrial Ca$^{2+}$ uptake, the permeabilized cells were supplemented with 0.5 mM fura2/FF/FA. Fluorescence was monitored in a multichannel length excitation dual wavelength emission fluorometer (Delta RAM, PTI) using 340 and 380 nm excitation and 535 nm emission for fura2FF, whereas 490 nm excitation/535 nm emission and 570 nm excitation/595 nm emission were used for JC-1. Cytosolic Ca$^{2+}$ is shown as the excitation ratio (340 nm/380 nm) of fura2FF/FA fluorescence, whereas ΔΨm is shown as the ratio of the fluorescence of J-aggregate (570 nm excitation/595 nm emission) and monomer (490 nm excitation/535 nm emission) forms of JC-1 (Szalai et al., 1999). All the experiments were performed at 35°C with constant stirring.

Analysis of cyto c distribution and caspase-3 activation by immuno blotting

At the end of the ΔΨm and [Ca$^{2+}$]i measurements in permeabilized cell suspensions, cells were immediately centrifuged at 14,000 g for 10 min and the supernatant collected as cytosol. The amount of protein present in the cytosol was measured using the Bradford method.

In studies with intact HepG2 cells, cells were grown in 25-cm$^2$ flasks for 5 h and were later treated with either xanthine 0.1 mM plus xanthine 20 mM/ml or in combination with DIDS (100 μM), CsA (5 μM), and BA (10 μM). At appropriate time periods, cells were harvested and washed in PBS. Cells were then resuspended in and permeabilized with 40 μg/ml digitonin in 1.0 ml of ICM supplemented with 1 μg/ml of each of antipain, leupeptin, and pepstatin and mitochondria (pellet) were stored at −80°C for Western blot analysis. For Western blot experiments, proteins (20–25 μg) were loaded onto each lane of a 15% SDS-PAGE and electrophoretically transferred to nitrocellulose filters. Filters were blocked with blocking buffer (Pierce Chemical Co.) overnight, followed by incubation with anti-cyto c (7H8.2C12; BD PharMingen) and anticaspase-3 antibody (C-20; Santa Cruz, CA), and horseradish peroxidase-conjugated secondary antibody and Dura Signal chemiluminescence–developing kit (Pierce Chemical Co.).

Fluorimetric measurement of caspase activity

DEVD-AMC cleavage was measured in cell extracts as described previously (Szalai et al., 1999).

DNA fragmentation analysis

Cells grown in 100-mm culture dishes (3 × 10$^6$ cells) were collected in their culture medium, pelleted at 125 g for 5 min, and pellets were washed twice with ice-cold PBS. Pellets were digested with 400 μl of digestion buffer (100 mM NaCl, 100 mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS, 20 μg/ml DNase-free RNase final concentration) for 60 min at 37°C. These homogenates were further incubated with proteinase K (0.1 mg/ml) for 15 min at 50°C. Supernatants were collected to a new tube, in which they were extracted with phenol/chloroform/isoamyl alcohol (25: 24:1). DNA was precipitated with 2 vol of cold ethanol and DNA was recovered after centrifugation at 500 g for 2 min. Precipitated DNA was washed once with 70% ethanol and resuspended in 1 mM EDTA, 10 mM Tris-HCl, pH 8.0. Isolated genomic DNA was resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Purification and incorporation of VDAC into liposomes and confocal imaging of cyto c translocation

Mitochondria isolated from rat liver were used for purification of VDAC as described previously (de Pinto et al., 1987). The purity of VDAC was assessed by SDS-PAGE electrophoresis and confirmed by Western blotting (antiporin 31HL antibody Calbiochem). Liposomes were prepared by soni- catizing 500 mg of phospholipid mixture (soybean Type II-S) in 10 ml of buffer containing 50 mM KCl, 20 mM KH$_2$PO$_4$, 20 mM Hepes and 1 mM EDTA, pH 7.0. After sonication, incorporation of purified VDAC and loading of cyto c-FITC (cyto c-FITC was purified as described in the labeling kit protocol [Molecular Probes] and purity was assessed by phosphor imager and Western blotting) was performed by subjecting these vesicles for two freeze–thaw cycles. For imaging of cyto c efflux, fluorescence of cyto c-FITC was monitored using a confocal system (BioRad-102/42, excitation 488 nm) fitted to an Olympus IX70 inverted microscope (60× objective). Images were taken from plain and VDAC incorporated liposomes before and 7 min after addition of X (100 μM) and XO (20 μM/ml). For quantitative analysis, liposomes were centrifuged and FITC was released to the supernatant and residual FITC in the liposomal fraction were determined fluorometrically (490 nm excitation/510 nm emission).

Electron microscopy

Naive and X + XO (0.1 mM plus 20 μM/ml)–treated cells were fixed in 2% glutaraldehyde, 1% tannic acid, 0.1 M sodium cacodylate at pH 7.4 for 2 h at room temperature. Processing, embedding of the fixed cells, cutting, staining, and examination of the sections (80-nm thick) were performed as described recently (Pacher et al., 2000).

Confocal imaging analysis of CCR and apoptotic markers in intact individual cells

For imaging of cyto c distribution, cells transfected with cyto c–GFP construct were treated with either X (0.1 mM) plus XO (20 μM) or in combination with DIDS (100 μM) or CsA (1 μM). After 3 h, cells were washed twice with 0.2% extracellular medium (2% BSA/ECD) consisting of 121 mM NaCl, 5 mM NaHCO$_3$, 10 mM NaHepes, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2 mM CaCl$_2$, 10 mM glucose, and 2% BSA, pH 7.4. The coverslips were transferred to the microscope stage and cyto c–GFP distribution was monitored with confocal imaging (488 nm excitation).

For imaging of phosphatidylserine exposure and ΔΨm, HepG2 cells were exposed to X + XO for various time periods in the absence of presence of DIDS (100 μM), then incubated with annexin V Alexa 488 for 15 min using 1× binding buffer (Molecular Probes). During the annexin incubation period, TMRE (50 nM) was also loaded (10 min) for the measurement of ΔΨm or propidium iodide (0.5 μg/ml) to evaluate the plasma membrane integrity.

Fluorescence recordings and confocal images are representative of three to six independent experiments. The data combined from separate experiments are shown as mean ±SE. Significance of differences from the relevant controls was calculated by Student’s t-test.
References


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