Selective role for superoxide in InsP₃ receptor–mediated mitochondrial dysfunction and endothelial apoptosis

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Reactive oxygen species (ROS) play a divergent role in both cell survival and cell death during ischemia/reperfusion (I/R) injury and associated inflammation. In this study, ROS generation by activated macrophages evoked an intracellular Ca²⁺ ([Ca²⁺]i) transient in endothelial cells that was ablated by a combination of superoxide dismutase and an anion channel blocker. [Ca²⁺]i store depletion, but not extracellular Ca²⁺ chelation, prevented [Ca²⁺]i elevation in response to O₂⁻ that was inositol 1,4,5-trisphosphate (InsP₃) dependent, and cells lacking the three InsP₃ receptor (InsP₃R) isoforms failed to display the [Ca²⁺]i transient. Importantly, the O₂⁻–triggered Ca²⁺ mobilization preceded a loss in mitochondrial membrane potential that was independent of other oxidants and mitochondrially derived ROS. Activation of apoptosis occurred selectively in response to O₂⁻ and could be prevented by [Ca²⁺]i buffering. This study provides evidence that O₂⁻ facilitates an InsP₃R–linked apoptotic cascade and may serve a critical function in I/R injury and inflammation.

Introduction

Receptor-mediated generation of reactive oxygen species (ROS) is necessary for signal transduction, gene expression, and cell proliferation in smooth muscle cells, T and B lymphocytes, and fibroblasts (Devadas et al., 2002). Conversely, ROS produced under pathological conditions such as ischemia/reperfusion (I/R) or inflammation are associated with cellular dysfunction and apoptosis (Davies, 1995). Endothelial cells respond to numerous external stimuli by producing the superoxide anion (O₂⁻). In physiological conditions, mitochondrial respiratory chain proteins produce O₂⁻, which can be dismutated into hydrogen peroxide (H₂O₂) or react with nitric oxide to produce peroxynitrite. In addition, reaction of H₂O₂ with iron leads to hydroxyl radical formation via Fenton chemistry. During I/R injury, O₂⁻ production in the vasculature is substantially increased (Wei et al., 1999) and is accompanied by endothelial cytotoxicity (for review see Li and Shah, 2004). However, the molecular mechanisms by which ROS lead to organ damage are poorly understood.

In pathological conditions, cell death is facilitated by an elevation in intracellular calcium ([Ca²⁺]i; Hajnoczky et al., 2003; Orrenius et al., 2003) via inositol 1,4,5-trisphosphate (InsP₃). InsP₃ is a second messenger produced by the hydrolysis of phosphatidylinositol-4,5-bisphosphate by PLC. InsP₃ receptor (InsP₃R)–mediated [Ca²⁺]i changes are associated with a rapid, transient Ca²⁺ release from Ca²⁺ stores in the ER followed by Ca²⁺ entry through slow-activating plasma membrane store-operated channels (Putney and Bird, 1993; Parekh and Penner, 1997; Berridge et al., 1998). InsP₃ [Ca²⁺]i signals control a wide range of cellular functions, including cell proliferation and apoptosis (Berridge et al., 2000; Orrenius et al., 2003). Apoptosis is reduced in cells lacking all three InsP₃R isoforms (DT40 avian B cells) and after selective suppression of InsP₃R-3 (Jayaraman and Marks, 1997; Sugawara et al., 1997), indicating the important role of InsP₃ in cell death mechanisms (Pan et al., 2001).

ALTERATION IN [Ca²⁺]i after oxidative stress facilitate...
activation of the mitochondrial permeability transition pore (MPTP), which releases cytochrome c from the mitochondrial intermembrane space, leading to mitochondrial membrane potential (ΔΨm) loss, assembly of the apoptosome, and activation of downstream caspases (Crompton, 1999). Recent evidence suggested that cytochrome c transiently released from mitochondria interacts with InsP₃R and amplifies Ca²⁺-mediated apoptosis (Boehning et al., 2003).

Endothelial cells subjected to oxidative stress undergo apoptosis (Warren et al., 2000). Although there is evidence that perturbations of cellular Ca²⁺ homeostasis (including [Ca²⁺]c elevation, ER Ca²⁺ depletion, and mitochondrial Ca²⁺ increases) occur, the mechanisms by which oxidative stress mediates endothelial apoptosis remain unclear. Events in the early stages of stress signaling include the mobilization of [Ca²⁺]c (Chen et al., 2004), in contrast to our findings that Bcl-X₁, which in turn controlled InsP₃R evoked Ca²⁺ release (Chen et al., 2004), in contrast to our findings that Bcl-X₁ activates InsP₃R (White et al., 2005). In addition, ER-localized Bax and Bak can either interfere with ER Ca²⁺ homeostasis or initiate apoptosis by activating caspase 12 (Zong et al., 2003).

We previously reported that cells exposed to O₂⁻, which is a key signaling molecule that coordinates multiple processes that lead to mitochondrial apoptotic events and endothelial dysfunction.

**Results**

**Lipopolysaccharide (LPS)-stimulated macrophages evoke Ca²⁺ transients in endothelial cells**

Activated macrophages are known to generate ROS and may be involved in organ damage during IR (Droge, 2002). To test the significance of the selective role of macrophage-derived ROS during pathophysiological conditions, LPS-stimulated murine macrophages were used as a O₂⁻-generating source. We determined whether O₂⁻ released from macrophages could evoke Ca²⁺ mobilization in two cell types, endothelial and HepG2 cells. ROS production in LPS-stimulated mouse macrophages was measured via H₂DCF-DA, which is a non-fluorescent dye that produces the fluorescent compound dichlorofluorescin (DCF) when oxidized by ROS. DCF fluorescence was measured in untreated macrophages and those stimulated with LPS (1 μg/ml) or a combination of LPS and the NADPH oxidase inhibitor diphenyleneiodonium (DPI; 30 μM). LPS stimulation was associated with a pronounced increase in DCF fluorescence that was attenuated by DPI treatment, suggesting that LPS-stimulated ROS production through activation of oxidative burst reactions (Fig. 1 A). The activation of macrophage NADPH oxidase generates O₂⁻ extracellularly without altering intracellular production of ROS by mitochondria (Lambeth, 2004). To elucidate whether a paracrine ROS signal can be transduced to adjacent cells in pathological conditions, LPS-stimulated macrophages were added onto pulmonary microvascular endothelial cells (PMVECs; Fig. 1 B) that had been previously loaded with the [Ca²⁺]c indicator dye Fluo-4 (Fig. 1 C). Application of LPS-activated macrophages evoked a
[Ca$^{2+}$], rise in PMVECs that was attenuated by DPI pretreatment (Fig. 1 C). To exclude the contribution of autocrine extracellular ROS production, a similar experiment was performed using HepG2 parenchymal cells, as these cells generate minimal O$_2^-$ (Kikuchi et al., 2000). HepG2 cells displayed an [Ca$^{2+}$], elevation after LPS-stimulated macrophage exposure, whereas no [Ca$^{2+}$] transient was noted after application of non-stimulated macrophages (Fig. 1 D). In contrast, exposure of HepG2 cells to macrophages that had been stimulated by LPS plus DPI triggered only an extremely small [Ca$^{2+}$], rise (Fig. 1 D). The oscillatory [Ca$^{2+}$], transient pattern observed in individual HepG2 cells but not PMVECs is notable, indicating a potential difference in Ca$^{2+}$ handling between cell types (unpublished data). Overall, this result suggests that O$_2^-$ is specifically required for elevation of [Ca$^{2+}$] in endothelial cells.

O$_2^-$ evokes endothelial Ca$^{2+}$ transients through InsP$_3$ signaling

To identify the mechanisms by which O$_2^-$ triggers Ca$^{2+}$ signals in PMVECs, we extended our studies to examine the effects of O$_2^-$ on basal [Ca$^{2+}$]. To exclude the possible contribution of other macrophage factors, the xanthine/xanthine oxidase (X+XO) system was used to generate O$_2^-$ externally. Cells exposed to O$_2^-$ demonstrated a rapid increase in [Ca$^{2+}$], followed by a slightly delayed return to baseline (Fig. 2 A). Similarly, the physiological stimulus ATP generated a marked [Ca$^{2+}$], transient (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200505022/DC1). The O$_2^-$-evoked [Ca$^{2+}$], increase was abolished by pretreatment with the XO inhibitor allopurinol (1 mM; Fig. 2 B) or by a combination of the antioxidant superoxide dismutase (SOD; 2000 U/ml) and the anion channel blocker DIDS (100 μM; Fig. 2 C). Treatment with either xanthine or allopurinol did not alter basal [Ca$^{2+}$], in control cells (unpublished data). These findings suggest that acute exposure of PMVECs to extracellular O$_2^-$ results in a rapid [Ca$^{2+}$], rise. We next sought to determine the source of the elevated [Ca$^{2+}$],. Thapsigargin (Tg) inhibits the SERCA Ca$^{2+}$-ATPase, causing Ca$^{2+}$ depletion from the ER (Ma et al., 2001; Bootman et al., 1999, 2000, 2001). Pretreatment with 2 μM Tg virtually abolished O$_2^-$-induced Ca$^{2+}$ transients (Fig. 2 D). Conversely, removal of Ca$^{2+}$ from the external medium was without effect on [Ca$^{2+}$], (Fig. 2 E). Together, these results indicate that O$_2^-$ induces a release of Ca$^{2+}$ from internal stores. ER Ca$^{2+}$ stores in endothelial cells can be modulated by production of the second messenger InsP$_3$ by PLC and subsequent binding to receptors on the ER (InsP$_3$R). To characterize the release of Ca$^{2+}$ from intracellular stores, PMVECs were pretreated for 10 min with either the PLC inhibitor U-73122 or its inactive analogue U-73343. U-73122, but not U-73343 (both 100 μM), inhibited the O$_2^-$-induced Ca$^{2+}$ release (Fig. 2 F and G). This result suggests that the O$_2^-$-induced [Ca$^{2+}$], transient was mediated by InsP$_3$. To further characterize O$_2^-$-induced Ca$^{2+}$ release, cells were incubated with 2-aminoethoxydiphenyl borate (2-APB; 75 μM) before O$_2^-$ stimulation. 2-APB has widely been used as an inhibitor of InsP$_3$-sensitive Ca$^{2+}$ release and store-operated Ca$^{2+}$ channels in intact cells (Ma et al., 2001; Bootman et al., 2002). In agreement with our PLC data, O$_2^-$-induced Ca$^{2+}$ transients were abolished in cells pretreated with 2-APB (Fig. 2, H and I). Thus, the O$_2^-$-induced [Ca$^{2+}$], rise in PMVECs was due to the InsP$_3$-dependent release of Ca$^{2+}$ from internal stores.

O$_2^-$-triggered [Ca$^{2+}$], release is abolished in InsP$_3$R triple knockout (TKO) cells

To examine the specific role of InsP$_3$R in the O$_2^-$-triggered [Ca$^{2+}$], rise, the InsP$_3$R-deficient DT40 chicken B-lymphocyte cell line (DT40 InsP$_3$R TKO) was used. Wild-type cells demonstrated a significant [Ca$^{2+}$], increase after O$_2^-$ exposure. After [Ca$^{2+}$], returned to basal levels, 2 μM Tg was added to the medium to induce a transient increase in [Ca$^{2+}$], as a consequence of passive depletion of endogenous stores upon ER Ca$^{2+}$/Mg$^{2+}$-ATPase blockade (Fig. 3, A and B). Similar to PMVECs, pretreatment with 2 μM Tg eliminated the O$_2^-$-induced Ca$^{2+}$ transients in wild-type DT40 cells (unpublished data). In contrast, addition of a O$_2^-$ pulse failed to elicit Ca$^{2+}$ release from intracellular stores in DT40 InsP$_3$R TKO cells, whereas subsequent addition of 2 μM Tg triggered a complete depletion of Ca$^{2+}$ stores (Fig. 3, A and B). These data suggest that Ca$^{2+}$ release through the InsP$_3$R underlies the O$_2^-$-evoked rise of [Ca$^{2+}$]. To confirm that DT40 InsP$_3$R TKO cells retain the machinery necessary for the O$_2^-$-mediated [Ca$^{2+}$], trans-
sient, we transfected the rat InsP3R type I into TKO cells. This procedure restored the responsiveness of TKO cells to O$_2^-$ (Fig. 3 C). This result indicates that in TKO cells, a O$_2^-$-mediated signal activates InsP3R type I and causes Ca$^{2+}$ release from ER store.

In PMVECs, inhibition of PLC with U-73122 prevented the rise of [Ca$^{2+}$], induced by exposure to O$_2^-$ (Fig. 3 A). We therefore further investigated the role of PLC in O$_2^-$-triggered Ca$^{2+}$ mobilization using PLC-γ2-deficient DT40 cells. O$_2^-$ exposure triggered a substantial rise of [Ca$^{2+}$] in PLC-γ2-deficient DT40 cells (Fig. 3, A and B). In wild-type DT40 cells, B cell receptor agonist IgM (2 μg/ml) induced a series of rapid [Ca$^{2+}$], oscillations representing [Ca$^{2+}$], release and reuptake. In contrast, anti-IgM failed to elicit Ca$^{2+}$ mobilization in both InsP3R TKO and PLC-γ2 knockout (KO) cells (unpublished data). These data indicate that the nonreceptor tyrosine kinase–linked cascade, to which PLC-γ2 is coupled, is dispensable for the O$_2^-$-triggered [Ca$^{2+}$] rise. In agreement with our findings, G protein–coupled receptor (GPRC)–mediated Ca$^{2+}$ oscillations were previously abolished by U-73122, which inhibits all PLC-β isoforms (Zeng et al., 2003). To further understand the role of InsP3, PLC-γ2 KO cells were pretreated with either PLC inhibitor U-73122 or U-73343 as described in Fig. 2 (F and G). U-73122, but not U-73343, attenuated the O$_2^-$-evoked [Ca$^{2+}$] rise (Fig. 3 D). To ensure that the O$_2^-$ elicits InsP3 accumulation, InsP3 was assayed in wild-type DT40, DT40 InsP3TKO, and DT40 PLC-γ2 KO cells. Direct measurement of InsP3 production indicated that O$_2^-$ markedly activated InsP3 formation in wild-type DT40, DT40 InsP3TKO, and DT40 PLC-γ2 KO cells. In contrast, pretreatment of DT40 PLC-γ2 KO cells with U-73122 significantly attenuated this response (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200505022/DC1). Similarly, PMVECs exposed to O$_2^-$ exhibited markedly greater InsP3 production than the physiological stimulus ATP (Fig. S2 B). Collectively, these findings suggest that extracellular O$_2^-$ causes Ca$^{2+}$ release via a PLC-mediated increase in InsP3.

O$_2^-$ mediates coupling of [Ca$^{2+}$], elevation and mitochondrial uptake

It is believed that agonist-induced [Ca$^{2+}$], can be buffered by mitochondria (Bernardi and Petronilli, 1996). To determine if the O$_2^-$-triggered [Ca$^{2+}$], spike is delivered to mitochondria, rhod-2– (mitochondrial Ca$^{2+}$ indicator) and Fluo-4–loaded PMVECs were subjected to O$_2^-$ exposure. Exposure of PMVECs to O$_2^-$ induced a [Ca$^{2+}$], increase as evidenced by an increase in Fluo-4 fluorescence, as shown earlier (Fig. 2 A), followed by an elevation of mitochondrial Ca$^{2+}$ fluorescence (Fig. 4, A and B). Similarly, ATP induced a [Ca$^{2+}$], rise followed by mitochondrial [Ca$^{2+}$] elevation (Fig. 4, C and D). These results indicate Ca$^{2+}$ signal propagation from the cytosol to the mitochondria in both physiological (purinergic receptor agonist) and pathological conditions (oxidative stress). Notably, O$_2^-$-evoked mitochondrial Ca$^{2+}$ elevation was increased and sustained compared with the transient pattern observed in response to ATP. These results suggest that O$_2^-$-induced intracellular pool Ca$^{2+}$ release evokes elevated mitochondrial Ca$^{2+}$ uptake during oxidative stress.
**O$_2^−$-induced Ca$^{2+}$ transients evoke rapid mitochondrial depolarization**

Reversible depolarization of ΔΨ$_m$ occurs as a consequence of electronic uptake of Ca$^{2+}$ by mitochondria in response to transient [Ca$^{2+}$]$_i$ (Duchen, 1992). However, ROS may also promote MPTP opening (Huser et al., 1998). Because mitochondrial Ca$^{2+}$ elevation is a common pathway in both normal physiological and pathological stimuli, we examined whether the observed mitochondrial Ca$^{2+}$ uptake after O$_2^−$ exposure is associated with mitochondrial depolarization. Simultaneous fluorescence measurements of [Ca$^{2+}$]$_i$, and ΔΨ$_m$ were conducted in PMVECs during O$_2^−$ exposure (Fig. 5, A and B). In response to ATP, an [Ca$^{2+}$]$_i$ rise was observed similar to that in cells after O$_2^−$ exposure. However, in contrast to O$_2^−$, PMVECs exposed to ATP exhibited only a nominal change in ΔΨ$_m$ (Fig. 5 C), possibly due to transient Ca$^{2+}$ uptake (Fig. 4, B and D). Application of O$_2^−$ evoked a rapid and transient rise in [Ca$^{2+}$]$_i$, that preceded a decrease in tetramethylrhodamine, ethyl ester, perchlorate (TMRE) fluorescence, indicating that mitochondrial depolarization is associated with the onset of the [Ca$^{2+}$]$_i$ rise (Fig. 5 B). Because O$_2^−$ is rapidly dismutated into H$_2$O$_2$, we sought to determine which oxidants are involved in the observed ΔΨ$_m$ loss. Cells incubated with H$_2$O$_2$ (1 mM) displayed no rapid [Ca$^{2+}$]$_i$ transient. Rather, H$_2$O$_2$ induced a slight increase in [Ca$^{2+}$]$_i$ (Fig. 5 D) and a delayed loss of ΔΨ$_m$. T2 pretreatment did not affect the H$_2$O$_2$-facilitated slow [Ca$^{2+}$]$_i$ rise (unpublished data). These findings suggest that H$_2$O$_2$ may not affect the intracellular store, but instead facilitates Ca$^{2+}$ entry from the extracellular milieu independent of mitochondrial depolarization. Oxidized phospholipid byproducts are involved in cell death during oxidative stress (Ran et al., 2004). However, the lipid-oxidizing agent t-butyl hydroperoxide (t-BuOOH; 200 μM) did not evoke either an [Ca$^{2+}$]$_i$ rise or ΔΨ$_m$ loss (Fig. 5 E). This finding suggests the selective role of O$_2^−$, and not other oxidants, in eliciting an [Ca$^{2+}$]$_i$ rise and mitochondrial depolarization.

**Extracellular O$_2^−$-mediated signaling functions independent of mitochondrially derived ROS**

Evidence indicates that external ROS may evoke mitochondrial O$_2^−$ production (Zorov et al., 2000; Aon et al., 2003). Because the O$_2^−$-evoked [Ca$^{2+}$]$_i$ rise is a prerequisite for ΔΨ$_m$ loss, we aimed to exclude the involvement of intracellular ROS production by mitochondrial electron transport proteins in ΔΨ$_m$ loss. Antimycin A inhibits the normal electron flow through complex III, but triggers O$_2^−$ production through the accumulation of ubisemiquinone. Antimycin A triggered an immediate ΔΨ$_m$ loss without an apparent change in [Ca$^{2+}$]$_i$ (Fig. 6 A). Rotenone inhibits electron transfer from complex I (NADH dehydrogenase) to ubiquinone and diminishes O$_2^−$ production from complex III (Turrens et al., 1985). In contrast to antimycin A, rotenone affected neither [Ca$^{2+}$]$_i$ nor ΔΨ$_m$. However, subsequent addition of O$_2^−$ triggered an [Ca$^{2+}$]$_i$ rise followed by ΔΨ$_m$ loss (Fig. 6 B). Oligomycin, which inhibits the mitochondrial F$_{1}$F$_{o}$-ATPase by binding to ATP synthase, was used to exclude possible mitochondrial ATP-dependent ROS production. Treatment with oligomycin failed to trigger either [Ca$^{2+}$]$_i$ mobilization or ΔΨ$_m$ loss. Subsequent addition of O$_2^−$ established both events...
Selective $O_2^-$ induction of Ca$^{2+}$ signaling evokes mitochondrial depolarization. (A) PMVECs loaded with Fluo-4/AM (30 min) and stained with TMRE (15 min) were exposed to $O_2^-$ as indicated (n = 8). (B) Relative brightness of Fluo-4 fluorescence and punctate–diffuse index of TMRE was calculated and plotted over time. (C) Change in $[Ca^{2+}]_i$ and $\Delta \Psi_m$, in response to 100 $\mu$M ATP (n = 3). [Ca$^{2+}$]i, and mitochondrial $\Delta \Psi_m$ were recorded in response to 1 mM $H_2O_2$ (D; n = 4) and 200 $\mu$M t-BuOOH (E; n = 4).

Figure 5. Selective $O_2^-$ induction of Ca$^{2+}$ signaling evokes mitochondrial depolarization. (A) PMVECs loaded with Fluo-4/AM (30 min) and stained with TMRE (15 min) were exposed to $O_2^-$ as indicated (n = 8). (B) Relative brightness of Fluo-4 fluorescence and punctate–diffuse index of TMRE was calculated and plotted over time. (C) Change in $[Ca^{2+}]_i$ and $\Delta \Psi_m$, in response to 100 $\mu$M ATP (n = 3). [Ca$^{2+}$]i, and mitochondrial $\Delta \Psi_m$ were recorded in response to 1 mM $H_2O_2$ (D; n = 4) and 200 $\mu$M t-BuOOH (E; n = 4).

methyl ester (25 $\mu$M for 30 min) before application of the $O_2^-$. BAPTA loading significantly inhibited $O_2^-$-induced $\Delta \Psi_m$ loss (Fig. 7, A and B). In contrast, the $H_2O_2$-induced $\Delta \Psi_m$ loss was unaffected by pretreatment with BAPTA (Fig. 7 C). These experimental data provide evidence that $\Delta \Psi_m$ loss induced specifically by $O_2^-$ requires a rise of [Ca$^{2+}$]i. Other oxidants such as $H_2O_2$ are deleterious to mitochondrial function but appear to affect $\Delta \Psi_m$ through a Ca$^{2+}$-independent pathway.

$O_2^-$-mediated signaling triggers caspase activation

Caspase cysteine proteases augment mitochondrial dysfunction by both activating proapoptotic Bcl-2 family proteins such as Bax, Bak, and Bid and inactivating antiapoptotic proteins such as Bcl-2 (Wei et al., 2001). To determine the dose and time course of receptor-mediated and mitochondrially dependent caspase activation in PMVECs after oxidant exposure, cytotoxic extracts were collected after treatment with $O_2^-$, $H_2O_2$, and t-BuOOH. Remarkably, when cells were exposed to $O_2^-$, robust caspase-3 activity was observed in a dose-dependent manner (Fig. 8, A and D). Interestingly, even a low dose (1 mU X+XO) was able to induce caspase-3 activity, indicating that $O_2^-$ may activate downstream caspases through a mitochondrially dependent pathway. Similarly, prominent caspase-9 activity was observed after $O_2^-$ treatment (Fig. 8, C and F). $H_2O_2$ elicited some caspase-3 and -9 activity, but at a level several-fold less than $O_2^-$. In contrast, t-BuOOH did not activate either

(Ca$^{2+}$ buffering protects against $O_2^-$-triggered mitochondrial depolarization

To assess whether the $O_2^-$-induced rise of [Ca$^{2+}$]i is required for the $O_2^-$-evoked $\Delta \Psi_m$ loss, PMVECs were loaded with the Ca$^{2+}$ chelator 1,2-bis(2-aminophenoxy)ethane-$N,N',N''$-tetraacetate (BAPTA) by incubation with the permeant acetoxy-
caspase-3 or -9. During apoptotic conditions, caspase-8 can activate caspase-3 directly through an extrinsic pathway. As shown in Fig. 8 (B and E), treatment of PMVECs with O$_2^-$/H$_2$O$_2$ induced caspase-8 activity that was sevenfold higher than control and other oxidants. Inhibition of O$_2^-$ loss by [Ca$^{2+}$]i buffering prevented caspase-3 and -9 activation (Fig. 8, D and F). Collectively, these results provide evidence that O$_2^-$ activates both extrinsic and intrinsic caspase pathways.

$O_2^-$-evoked [Ca$^{2+}$]i overload executes the cell death machinery

Our results reveal that O$_2^-$ stimulates [Ca$^{2+}$], mobilization that triggers subsequent mitochondrial events, leading to caspase activation in PMVECs. To directly demonstrate that O$_2^-$ induces apoptosis, we treated PMVECs with various oxidants at different doses, and then stained them for the early apoptotic marker annexin V and the late stage apoptotic (or necrotic) marker propidium iodide (PI). Cells treated with O$_2^-$ for 5 h displayed positive annexin V staining with no detectable PI labeling, indicating cells in the early stages of apoptosis (Fig. 9 A). Strikingly, cells exposed to a high concentration of O$_2^-$ demonstrated a dose-dependent elevation of both apoptotic and necrotic cell death as displayed in Fig. 9 B. Cells treated with 500 µM H$_2$O$_2$ also revealed an apoptotic phenotype, although at a lower level than observed in response to O$_2^-$. In contrast, t-BuOOH (200 µM) treatment primarily led to necrosis, as evidenced by positive annexin V and PI staining. Control conditions resulted in nominal levels of apoptotic- or necrotic-positive cells. Previously, our results provided evidence that buffering of O$_2^-$ evoked [Ca$^{2+}$]i rise by BAPTA-AM and markedly prevented PMVEC ΔΨ$_m$ loss. Therefore, we tested whether [Ca$^{2+}$]i buffering inhibits O$_2^-$-induced apoptosis. BAPTA-AM pretreatment (25 µM) attenuated apoptosis in PMVECs (Fig. 9 C), providing evidence that O$_2^-$-induced [Ca$^{2+}$]i elevation is essential for mitochondrial-dependent apoptosis. Conversely, BAPTA-AM treatment was ineffective 20 min after application of the O$_2^-$ (unpublished data). DT40 B-cells lacking all forms of InsP$_3$R display reduced apoptotic cell death in response to anti-IgM

Figure 7. Buffering of $O_2^-$-evoked [Ca$^{2+}$]i elevation with BAPTA prevents Ca$^{2+}$-induced ΔΨ$_m$ in PMVECs. (A and B) $O_2^-$-induced [Ca$^{2+}$]i elevation and ΔΨ$_m$ was prevented by pretreatment of cells with 25 µM of the membrane-permeable Ca$^{2+}$ chelator (BAPTA-AM; n = 4). (C) Chelation of intracellular Ca$^{2+}$ using BAPTA failed to attenuate H$_2$O$_2$-induced ΔΨ$_m$ (n = 4).

Figure 8. $O_2^-$-dependent activation of caspases in PMVECs. Cells were exposed to various concentrations of $O_2^-$, H$_2$O$_2$, and tBuOOH. After 3 h of treatment, lysates were assessed for caspase-3 (n = 3; A), -8 (n = 3; B), and -9 (n = 3; C) activity. Time-dependent experiments were also performed for caspase-3 (n = 3; D), -8 (n = 3; E), and -9 (n = 3; F). Pretreatment with 25 µM BAPTA-AM for 30 min attenuated caspase-3, -8, and -9 (n = 3) activation in response to $O_2^-$ as indicated in D, E, and F. Control cells were treated with 25 µM BAPTA-AM alone. Data are means ± SEM.
(Sugawara et al., 1997). Because O$_2^-$-induced [Ca$^{2+}$]$_i$ elevation is ablated in DT40 InsP$_3$R TKO cells, we next investigated apoptosis in DT40 cells. DT40 InsP$_3$R TKO cells, but not wild-type cells, display increased resistance to apoptosis after O$_2^-$ application (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200505022/DC1). These results suggest that O$_2^-$ selectively alters ER Ca$^{2+}$ homeostasis resulting in caspase activation, which in turn leads to apoptosis.

Discussion

The mechanisms that contribute to apoptosis during I/R injury remain unclear, but it is generally believed that the release and/or activation of various bioactive molecules, such as ROS (Zhao, 2004) and inflammatory cytokines (Haimovitz-Friedman et al., 1997), are responsible for cell death. During these conditions, xanthine (Malis and Bonventre, 1986) and NADPH oxidases play a key role in O$_2^-$ production (Wei et al., 1999) and trigger pathological signaling. Reperfusion of ischemic cells generates oxidative stress and alters mitochondrial function (Hausenloy et al., 2004). Coordination of mitochondrial function during injury is an essential component of cell physiology and survival, yet little is known about the factors that contribute to cell death during oxidative stress. This study demonstrates that O$_2^-$ facilitates a transient [Ca$^{2+}$]$_i$ elevation followed by mitochondrial Ca$^{2+}$ uptake and depolarization that ultimately induces apoptotic cascades in endothelial cells.

Macrophage activation by endotoxin elicits O$_2^-$ generation via NADPH oxidase and autocrine production of ROS (Johnston et al., 1978). However, whether released O$_2^-$ has a potential paracrine signaling role in nearby cells is unknown. This study provides direct evidence that activated macrophages initiate an ROS-induced [Ca$^{2+}$]$_i$ elevation in adjacent cells. In comparison to the macrophage data, the observed [Ca$^{2+}$]$_i$ transient using the XOX was larger and less sustained. The enzymatic XOX system generates only O$_2^-$, whereas activated macrophages may release other factors that could alter the amplitude of [Ca$^{2+}$]$_i$ in PMVECs. In addition, xanthine oxidase has been shown to interact with the vascular endothelium during inflammatory conditions (Houston et al., 1999). Because of the short half-life of the O$_2^-$ radical, close association between endothelial cells and the O$_2^-$ source may facilitate a greater response. A single pulse of O$_2^-$ evoked an [Ca$^{2+}$]$_i$ rise in PMVECs that caused $\Delta\Psi_m$ loss. These results suggest a potential mechanism by which macrophage-mediated oxidative stress perpetuates endothelial dysfunction. This O$_2^-$-mediated response has several features. The [Ca$^{2+}$]$_i$ signals were observed in adherent PMVECs, HepG2, and DT40 suspension cell types, indicating a
common mechanism in the cellular response to $O_2^-$: The $O_2^-$-evoked $[Ca^{2+}]_{i}$ signal was prevented by the combination of SOD and the anion channel blocker DIDS. The $O_2^-$-induced transient rise of $[Ca^{2+}]_{i}$, was propagated to mitochondria, where a sustained $Ca^{2+}$ elevation was observed. In contrast, the $[Ca^{2+}]_{i}$ response to the physiological stimulus ATP triggered a transient mitochondrial $Ca^{2+}$ elevation. The $O_2^-$-induced $[Ca^{2+}]_{i}$ transient subsequently evoked mitochondrial depolarization independent of mitochondrially derived ROS. In addition to this novel observation, our results suggest that $O_2^-$ selectively evokes $Ca^{2+}$-dependent $\Delta$ψm loss independent of other oxidants.

Another important finding is that Tg, but not EGTA, pretreatment eliminated the $O_2^-$-induced increase in $[Ca^{2+}]_{i}$, indicating release from the ER. We therefore conclude that $Ca^{2+}$ store release in response to $O_2^-$ may be PLC dependent and mediated by InsP3R on the ER. This conclusion was supported by the observation that DT40 cells lacking all three InsP3R isoforms failed to show an $[Ca^{2+}]_{i}$ rise after $O_2^-$ application, unless InsP3R was reintroduced by transient transfection. Re-introduction of InsP3R type 1 restored the $[Ca^{2+}]_{i}$ transient, indicating the existence of the $Ca^{2+}$ signaling machinery in TKO cells. Furthermore, we found that the PLC inhibitor U-73122 blocked the $O_2^-$ response in endothelial cells. PLC normally presents as a key enzyme in cellular metabolism and signaling in response to extracellular agonists by coupling with GTP-binding proteins. DT40 cells express PLC-γ2 and PLC-β isoforms (Rhee, 2001) but lack the GPCRs necessary for PLC-β activation (Venkatachalam et al., 2001; Patterson et al., 2002). Surprisingly, we observed that PLC-γ2 KO cells displayed a rapid $[Ca^{2+}]_{i}$ store release in response to $O_2^-$, suggesting the activation of PLC-β-mediated $Ca^{2+}$ release by $O_2^-$. PLC inhibition in these PLC-γ2 KO cells by U-73122 indicates activation of PLC and suggests that $O_2^-$-induced $[Ca^{2+}]_{i}$ rise requires InsP3. Because InsP3 levels were greatly elevated by $O_2^-$ in all three DT40 cell lines, it is apparent that generation of InsP3 by PLC is the essential signal in response to $O_2^-$ for InsP3R activation. $Ca^{2+}$ release via PLC-β (Liao et al., 1989) was investigated using the G protein–coupled muscarinic M5 receptor agonist carbachol. No detectable $Ca^{2+}$ signals were observed in response to carbachol (500 µM), indicating that DT40 cells lack the GPCR machinery necessary for PLC-β activation (unpublished data). However, we cannot exclude that $O_2^-$ may directly activate signaling upstream of PLC or regulate InsP3R. Earlier, we demonstrated the activation of mitochondrial PLA2 by $O_2^-$ (Madesh and Balasubramanian, 1997), lending support to our findings on the activation of signaling enzymes by $O_2^-$.

Our findings suggest that $\Delta$ψm loss in response to $O_2^-$ is dependent on ER stores and not extracellular $Ca^{2+}$. However, it is unclear whether mitochondrially derived ROS exacerbate $Ca^{2+}$ release from ER stores during oxidative stress. Rotenone and other distal complex I inhibitors generate $O_2^-$ on the matrix side of the inner membrane (Brookes et al., 2004). Our data indicate that cells pretreated with rotenone alone did not trigger either $[Ca^{2+}]_{i}$ changes or a $\Delta$ψm change. In contrast, the complex III inhibitor antimycin A caused a sharp decline in the $\Delta$ψm without concomitant $[Ca^{2+}]_{i}$ mobilization. This finding suggests that $O_2^-$ generation by complex III directly facilitates $\Delta$ψm loss independent of $[Ca^{2+}]_{i}$ levels. Cell death can be initiated by mitochondrial inhibitors through a reduction in ATP levels in a process known as necrosis. Specifically, oligomycin is known to reduce available ATP through inhibition of mitochondrial F$_{1}$/F$_{0}$ -ATPase and to elicit cell death through a switch from apoptosis to necrosis. In our system, endothelial cells pretreated with oligomycin did not experience either a rapid $[Ca^{2+}]_{i}$ change or $\Delta$ψm decay. However, subsequent delivery of $O_2^-$ perturbed the ER $Ca^{2+}$ level and subsequent $\Delta$ψm loss. Experiments using the mitochondrial uncoupler FCCP indicate that mitochondrial $Ca^{2+}$ efflux precedes $\Delta$ψm dissipation. Apparently, mitochondrial depolarization evoked by pararicine $O_2^-$ differs from $\Delta$ψm alterations induced by mitochondrially derived ROS.

The question arises whether extracellular $O_2^-$ generation evokes selective signaling during endothelial dysfunction. Previously, cells exposed to $O_2^-$ but not $H_2O_2$ elicited a rapid and large cytochrome c release from the mitochondria, followed by $\Delta$ψm loss (Madesh and Hajnoczyk, 2001). Cell death has been associated with elevation of $Ca^{2+}$ through various means. Moreover, elevation of $[Ca^{2+}]_{i}$, has been implicated in the induction of apoptosis by ROS (Orrenius et al., 2003). It is suggested that $H_2O_2$ facilitates $Ca^{2+}$ entry from the extracellular milieu or from the intracellular pools (Zhao, 2004), and $H_2O_2$-induced apoptosis in I/R injury has also been proposed (Inser et al., 2000). This study suggests that $O_2^-$, but not $H_2O_2$, evoked an intracellular $Ca^{2+}$ release that regulates the $\Delta$ψm. Strikingly, pretreatment with the $[Ca^{2+}]_{i}$ chelator BAPTA-AM prevents $O_2^-$ but not $H_2O_2$-mediated endothelial $\Delta$ψm loss. Thus, the $O_2^-$-initiated $\Delta$ψm loss is dependent on an $[Ca^{2+}]_{i}$ rise and independent of mitochondrial ROS generation. These findings suggest that extracellularly generated $O_2^-$ rapidly evokes the observed $[Ca^{2+}]_{i}$ elevation and pathological $\Delta$ψm loss. Interestingly, we illustrate that externally delivered $O_2^-$, and not other oxidants, triggers a cytosolic signal that initiates the mitochondrial phase of apoptosis.

Mitochondrial membrane permeabilization evoked by apoptotic stimuli facilitate apoptogenic protein release from the intermembrane space and can lead to the downstream activation of both caspase-dependent and -independent apoptotic cascades. Our previous observation proposed that $O_2^-$, but not $H_2O_2$, elicited cytochrome c release via a voltage-dependent anion channel–dependent mitochondrial membrane permeabilization (Madesh and Hajnoczyk, 2001). Cytochrome c release is regulated by the Bcl-2 family of proteins, and the target of these proteins in the cell is the MPTP (Kroemer and Reed, 2000; Mattson and, Kroemer, 2003). This study shows the activation of initiator and effect caspases by $O_2^-$ specifically, and to some extent, by high doses of $H_2O_2$. Recent evidence has indicated that a caspase-3–truncated InsP3R type I may elicit a prolonged $[Ca^{2+}]_{i}$ elevation during apoptosis (Assefà et al., 2004). Our model indicates that caspase-3 activation is downstream of $[Ca^{2+}]_{i}$, elevation and $\Delta$ψm loss. However, we cannot rule out modification of InsP3R type I in the late stages of $O_2^-$-triggered apoptosis. Collectively, these findings establish that ER $Ca^{2+}$ mobilization is upstream of mitochondrial events evoked by $O_2^-$ in endothelial apoptosis.
In conclusion, activated macrophage-derived O$_2^-$ acts as an important signaling molecule that mediates Inp$_x$P$_x$R-linked [Ca$^{2+}$]-, elevation and mitochondrial dysfunction in endothelial cells and provides a novel signaling link between inflammatory and endothelial cells under pathological conditions. We therefore propose that paracrine O$_2^-$ signaling is critical to endothelial cell death.

Materials and methods

Cell culture
Primary rat PMVECs (provided by T. Stevens, University of South Alabama, Mobile, AL) were cultured in DME supplemented with 10% FBS, nonessential amino acids, and antibiotics. Cells of wild-type DT40 chicken B cell line, triple Inp$_x$P$_x$R KO cell line (DT40 Inp$_x$P$_x$R KO), and PLC-$\gamma$2 KO (provided by A. August, Pennsylvania State University, Philadelphia, PA) cell line were cultured in RPMI 1640 supplemented with 10% FCS, 1% chicken serum, 50 m$\mu$M 2-mercaptoethanol, 4 m$\mu$M glutamine, and antibiotics. J774A.1 monococyte-derived mouse macrophages were cultured in Hank’s F12 (supplemented with 10% FBS) and antibiotics. Heptocellular carcinoma cell line (HepG2) was cultured in MEM with 10% FBS, 2m$\mu$M-glutamine, 0.50 mM sodium pyruvate, 0.1 mM nonessential amino acids, and antibiotics. Cells between passages 2 and 10 were used for experiments.

Visualization of ROS generation
J774.1 mouse monocyte-derived macrophages (10$^5$ cells/ml) were cultured on glass bottom 35-mm dishes (Harvard Apparatus) for 48 h. Cells were challenged with 1$\mu$g/ml LPS for 3 h at 37°C. For DPI treatment, 2.5 h LPS-treated macrophages were incubated with 30$\mu$M DPI for 30 min. The oxidation-sensitive dye H$_2$DCF-DA (10$\mu$M; Invitrogen) was added separately to dishes 20 min before visualization under confocal microscopy. Macrophage cells treated under similar conditions were used for co-culture model Ca$^{2+}$-mobilization.

[Ca$^{2+}$]i measurement
Measurement of [Ca$^{2+}$]i changes was performed using the Ca$^{2+}$-sensitive fluorescent dye Fluo-4/AM (Invitrogen). Cells adherent to 25-mm-diam glass coverslips were incubated at RT in extracellular membrane (ECM) containing 5 m$\mu$M Fluo-4/AM for 30 min, followed by an additional 10-min incubation in a dye-free medium. Coverslips were affixed to a chamber and mounted in a PMM-2 open perfusion microincubator (Harvard Apparatus) and maintained at 37°C on an inverted microscope (model TE300; Nikon). Confocal imaging was performed using the Radiance 2000 imaging system (Bio-Rad Laboratories) equipped with a Kr/Ar-ion laser source at 488-nm excitation using a 60$\times$ oil objective. Images were collected using LaserSharp software (Bio-Rad Laboratories) every 3 s for [Ca$^{2+}$]i changes. Mobilization was induced by the application of 100 m$\mu$M and 5 m$\mu$M of, respectively, xanthine/xanthine oxidase O$_2^-$-generating system. Whole cell masking was used to quantitate individual cell responses (SpectraLyzer, custom software; provided by Paul Anderson, Thomas Jefferson University, Philadelphia, PA).

Measurement of inositol phosphates
24 h before experiments, cells (10$^5$/ml) were transferred to myo-inositol-free DME and incubated in the presence of myo-[2$^{-3}$H]inositol (2 m$\mu$Ci/ml; 20 Ci/mmol; MP Biomedical, Inc.). After washing with myo-inositol-free DME, cells were incubated for 30 min in myo-inositol-free DME supplemented with 10 mM LiCl and then exposed to either ATP (100 m$\mu$M) or X+XO (100 m$\mu$M xanthine and 5 m$\mu$M XO) for 20 min at 37°C. The medium was subsequently removed and cells were scraped into 1 ml of 10% (wt/vol) TCA for the extraction of soluble inositol phosphates. After centrifugation of the cell lysates, the supernatant was applied to AG 1-X8 (for sample form) ion exchange columns (200-400 mesh; Bio-Rad Laboratories). These columns were washed as previously described (Takata et al., 1995). Elution was performed with increasing concentrations of ammonium formate (0.1-0.7 M).

Simultaneous confocal imaging of cytosolic and mitochondrial Ca$^{2+}$ in PMVECs
Endothelial cells were loaded with 2 m$\mu$M rhod-2/AM in ECM containing 2.0% BSA in the presence of 0.003% pluronic acid at 37°C for 50 min. Cells loaded with rhod-2 dye were washed and then reloaded with Fluor-4/AM for an additional 30 min at RT. Cells were placed on a temperature-controlled stage and images were recorded using the Radiance 2000 imaging system with excitation at 488 and 568 nm for Fluo-4 and rhod-2, respectively.

Kinetics of [Ca$^{2+}$]i elevation and mitochondrial membrane depolarization
Cells cultured on 25-mm-diam glass coverslips were loaded for 30 min with 5 m$\mu$M Fluo-4/AM at RT. The cationic potentiometric fluorescent dye TMRE (100 nM) was added to the loading medium and allowed to equilibrate for at least 15 min. Under these conditions, TMRE fluorescence was largely localized to the mitochondrial matrix space. After dye loading, the cells were washed and resuspended in the experimental imaging solution (ECM containing 0.25% BSA). Intracellular esterase action then resulted in loading of both the cytoplasmic and mitochondrial compartments of the cell. Experiments were performed in ECM containing 0.25% BSA at 37°C. Images were recorded using the Radiance 2000 imaging system with excitation at 488 and 568 nm for Fluo-4 and TMRE, respectively. Fluo-4 and TMRE fluorescent changes were determined by background subtraction followed by masking of total cell area or intracellular regions. During ΔΨm loss, the exit of TMRE from mitochondria into the cytoplasm leads to quenching of the dye. The rapid redistribution of TMRE into the cytoplasm after depolarization of ΔΨm can be transiently detected in the nucleus.

Detection of caspase-3, -8, and -9 activity
The assay is based on the ability of the active enzymes to cleave the fluorogenic substrates Ac-DEVD-AFC (caspase-3), Ac-IETD-AFC (caspase-8), or Ac-LEHD-AFC (caspase-9; Calbiochem). Cells treated with various oxidants were harvested by trypsinization and washed with PBS. The cell pellet was gently resuspended in lysis buffer (25 mM Hepes, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, and protease inhibitor cocktail [Roche], lysed, and centrifuged; the supernatant was used as the assay. Caspase substrates were added to a final concentration of 50 μM and the samples were incubated at 37°C for 45 min in caspase assay buffer. Incubated samples were measured at an excitation of 400 nm and an emission of 505 nm in a multichannel-excitation dual wavelength-emission fluorometer (Delta-RAM; Photon Technology International).

Confocal imaging analysis of apoptotic markers in PMVECs
To determine cellular outcome in response to oxidative stress, cells were exposed to the O$_2^-$-generating system, H$_2$O$_2$, and H$_2$O$_2$OH for 5 h. To assess the externalization of phosphatidylserine in the plasma membrane, as occurs in the early stage of apoptosis, cells were incubated with the conjugate annexin V Alexa Fluor-488 (Invitrogen) and PI (0.5 μM) for 15 min. After treatment, annexin V- and PI-stained cells were visualized and counted. In normal cells, impermeable PI is internalized as the plasma membrane loses integrity. Thus, positive PI staining indicates either late stage of apoptosis or necrosis.

Data analysis
Tracings are representative of the mean fluorescence value of all cells in one field and are indicative of n independent experiments. Data given are representative of duplicate analysis of n independent experiments as mean ± SEM.

Online supplemental material
Fig. S1 shows the Ca$^{2+}$ response to the physiological and pathological stimuli ATP and O$_2^-$, respectively, in PMVECs. Fig. S2 details the measurement of Inp$_x$P$_x$R generation in both DT40 and PMVECs. Fig. S3 shows the analysis of apoptosis in DT40 cells in response to O$_2^-$-O$_2$OH. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200505022/DC1.

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