Mitochondrial function is a critical determinant of IL-1-induced ERK activation

Qin Wang,* Gregory P. Downey,† Maite Abreu,† Elena Bajenova,* András Kapus,‡ and Christopher A. McCulloch*

*CIHR Group in Matrix Dynamics, University of Toronto, Toronto, Ontario, Canada; †Division of Respirology, Department of Medicine, University of Toronto and the Research Institute, Toronto General Division of the University Health Network Research Institute, Toronto, Ontario, Canada; and ‡Department of Surgery, University of Toronto and the Division of Surgery, Toronto General Hospital, Toronto, Ontario, Canada

Corresponding author: C.A. McCulloch Room 244, Fitzgerald Building, 150 College Street, University of Toronto, Toronto, Ontario, CANADA M5S 3E2. E-mail: christopher.mcculloch@utoronto.ca

ABSTRACT

Interleukin-1 (IL-1) is a potent, proinflammatory cytokine, but local environmental factors in inflamed sites or in sepsis may affect cell metabolism and energetics, including the amplitude and duration of IL-1-induced signals, thereby leading to loss of tissue homeostasis. Currently, the mechanisms by which disruption of cell energetics affects inflammatory signaling are incompletely understood. Here, we examined the impact of cell energetics and mitochondrial function on the regulation of IL-1-induced Ca2+ signals and ERK activation in human gingival fibroblasts, cells that are important targets for IL-1-induced destruction of extracellular matrix in inflamed connective tissues. In untreated cells, IL-1 induced a prolonged increase of free intracellular calcium, which was required for ERK activation. Inhibition of cellular energetics by selective depolarization of mitochondria blocked Ca2+ uptake and almost completely abolished IL-1-induced cytosolic Ca2+ signals and ERK activation. IL-1 caused rapid Ca2+ release from the endoplasmic reticulum (ER), concomitant with mitochondrial Ca2+ uptake from ER and non-ER stores. Disruption of mitochondrial energetics abrogated IL-1 induced Ca2+ release from the ER but left other vital cellular functions intact. The negative effect of mitochondrial depolarization on ER release was bypassed by BAPTA/AM, indicating that mitochondrial Ca2+ buffering is the key mechanism in regulating ER release. Thus, in gingival fibroblasts, mitochondrial Ca2+ uptake is essential not only for shaping the kinetics and duration, but also the generation of, IL-1-induced Ca2+ signals. Consequently, mitochondria regulate key downstream effectors of IL-1, including MAP kinases.

Key words: Ca2+ signals • gingival fibroblasts • mitochondria

Interleukin-1 (IL-1) has been directly implicated in the destruction of extracellular matrices in several high-prevalence diseases, including rheumatoid arthritis, periodontitis, and cancer (1–4). IL-1 mediates degradation of extracellular matrix proteins by promoting the synthesis and
secretion of proteases and the release of reactive oxygen species that are expressed by several resident cell types in connective tissues, including macrophages and fibroblasts. The activation of matrix-degrading genes in these cells is critically dependent on the amplitude and duration of IL-1-induced signals (5, 6), particularly those associated with mitogen-activated protein (MAP) kinases (7, 8). Currently, the impacts of local environmental factors on those intracellular pathways that regulate the kinetics of IL-1-dependent gene expression are not defined. Notably, the impact of sepsis and inflammation on IL-1-induced signals has not been described. The bioenergetic failure that is characteristic of advanced inflammatory lesions and sepsis directly involves loss of mitochondrial function (9), including mitochondrial ion transport and calcium regulation (10), suggesting the possibility that mitochondrial regulation of IL-1-induced signals may be important in the pathophysiology of inflammation.

Mitochondrial calcium transport has a dual function: 1) it relays the cytosolic Ca\(^{2+}\) signal to the mitochondrial matrix, thereby enhancing ATP synthesis in stimulated cells; 2) it can contribute to the regulation of cytosolic Ca\(^{2+}\) transients. Mitochondria can take up large amounts of free Ca\(^{2+}\) after increases of [Ca\(^{2+}\)] in physiological conditions (11, 12), a process that includes the uptake of Ca\(^{2+}\) released from intracellular stores (13). This property may be important for IL-1-induced gene expression, as previous data have shown that frequency modulation by calcium signals can regulate the expression of c-fos (14, 15), a transcription factor required for the expression of IL-1-inducible genes, including collagenase-1 (16, 17).

The spatio-temporal properties of calcium signals are critical for regulating IL-1-induced downstream events, including activation of ERK (18, 19), a MAP kinase required for c-fos expression (20). Calcium-mobilizing agonists such as IL-1 typically stimulate an increase in [Ca\(^{2+}\)], with defined temporal characteristics. For example, cells expressing high levels of IL-1 type 1 signaling receptors, such as gingival fibroblasts (21), exhibit an early release of Ca\(^{2+}\) from intracellular stores after IL-1 stimulation (22). This is followed by a sustained increase of [Ca\(^{2+}\)], that is mediated by store-operated Ca\(^{2+}\) influx (19). The spatio-temporal properties of a particular Ca\(^{2+}\) signal are determined by a finely tuned interplay between various Ca\(^{2+}\)-regulating organelles. In this context, the relationship between the endoplasmic reticulum (ER) and store-operated Ca\(^{2+}\) channels in the plasma membrane (23–25), as well as the impact of the ER-plasma membrane crosstalk on temporal aspects of Ca\(^{2+}\) signaling, have been well documented (26). In addition, recent work has suggested a functional link between mitochondria, the ER, and activation of store-operated Ca\(^{2+}\) entry (27–36).

In view of these data and our lack of understanding of the role of mitochondria in regulating IL-1-induced signals, we determined the impact of mitochondrial energetics on release of Ca\(^{2+}\) from the ER, store-operated Ca\(^{2+}\) entry and on IL-1-induced ERK activation. Our data show that mitochondria play a central role in regulating IL-1-induced Ca\(^{2+}\) signals and downstream effectors, including the MAP kinase, ERK. These results indicate that Ca\(^{2+}\) uptake by functional mitochondria is a prerequisite for the generation of IL-1-induced Ca\(^{2+}\) signals in gingival fibroblasts. Moreover, our findings provide novel insights into how compartmentalization of signaling determines the responses of cells to inflammatory mediators such as IL-1.
MATERIALS AND METHODS

Materials

Ionomycin, latrunculin B, thapsigargin, antimycin A, oligomycin, FCCP, carbamoylcholine chloride (carbachol) and prostaglandin E2 were obtained from Sigma (St. Louis, MO). Rabbit polyclonal antibodies to ERK1/2 and JNK and mouse monoclonal anti-phospho-ERK1/2 and anti-phospho JNK were purchased from New England Biolabs (Beverly, MA). Horseradish peroxidase-conjugated goat anti-mouse (H+L) and goat anti-rabbit (H+L) were purchased from Cedarlane Laboratories (Hornby, ON). The ECL Chemiluminescence Kit was purchased from Amersham Life Science (Oakville, ON). Recombinant human IL-1β was obtained from R and D Systems (Minneapolis, MN). Fura-2/AM, mag-fura-2/AM, rhod-2 and BAPTA/AM were obtained from Molecular Probes (Eugene, OR). 2-APB was obtained from Calbiochem (San Diego, CA).

Cell culture

Human gingival fibroblasts (5th-12th passages) were grown in minimal essential medium (MEM) containing 10% fetal bovine serum and antibiotics (0.17% penicillin V, 0.1% gentamycin sulfate, and 0.01% amphotericin) in a humidified atmosphere of 5% CO2 in air. For all experiments except where indicated, cells were plated on fibronectin-coated substrates.

ATP levels

Cells were plated at a density of 103 – 105 cells per well in 12 well plates. Cellular ATP levels were quantified with a luciferin and luciferase-based assay. After 24–48 h, cells were washed with PBS and lysed with nucleotide-releasing buffer (100 µl) for 5 min at room temperature with gentle shaking. ATP monitoring enzyme (1 µl) was added to the cell lysate and samples were analyzed in a luminometer within 1 min.

Immunoblotting

Protein concentrations of cell lysates were determined by the Bradford assay (Bio-Rad). Equal amounts of protein were loaded on to SDS-polyacrylamide gels (10%), resolved by electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4°C in Tris-buffered saline solution with 5% milk to block nonspecific binding sites and incubated with primary antibodies for 1–4 h at room temperature in Tris-buffered saline with 0.1% Tween-20. Horseradish peroxidase secondary antibodies were incubated for 1 h at room temperature in Tris-buffered saline with 0.1% Tween-20 and 5% milk. Labeled proteins were visualized by chemiluminescence.

Calcium measurements

For measurement of whole cell [Ca2+]i, cells on fibronectin-coated cover slips were loaded with 3 µM fura-2/AM for 20 min at 37°C and measured by ratio fluorimetry as described (22,31). For estimation of [Ca2+]ER, cells were incubated with mag-fura-2/AM (4 µM) for 150 min at 37°C, in α-MEM containing 10% fetal bovine serum. The nominally calcium-free buffer consisted of a...
bicarbonate-free medium containing 150 mM NaCl, 5 mM KCl, 10 mM D-glucose, 1 mM MgSO₄, 1 mM Na₂HPO₄, and 20 mM HEPES at pH 7.4 with an osmolarity of 291 mosMol. For experiments requiring external Ca²⁺, 2 mM CaCl₂ was added to the buffer; for experiments requiring chelation of external Ca²⁺, 1 mM EGTA was added. After incubation with fura-2/AM, inspection of cells by fluorescence microscopy demonstrated no vesicular compartmentalization of fura-2, suggesting that the dye loading method permitted measurement of cytosolic [Ca²⁺]. Visual inspection of mag-fura-2-loaded cells showed fluorescent labeling of intracellular organelles and as shown earlier (31), these labeled organelles correspond to the ER. Whole cell [Ca²⁺], measurements and store-specific [Ca²⁺] were obtained with C•IMAGING SYSTEMS–Simple PCI software (Compix, Cranberry Township, PA) with excitation wavelengths of 340 and 380 nm and an emission wavelength of 520 nm. Changes in [Ca²⁺], or for specific compartments were monitored by the ratio of fura-2 fluorescence at 340 and 380 nm.

For analysis of mitochondrial calcium, fibroblasts were loaded with 4.5 µM rhod-2/AM in 0.005% (v/v) Pluronic F-127 gel for 30–140 min at 37°C (31). The cells were washed twice and incubated in calcium buffer for imaging. The magnitude of fluorescence of rhod-2-stained samples was observed in a Nikon inverted microscope equipped with a CCD camera (Orca, Hamamatsu) and analyzed with Compix software. As ratio imaging cannot be used to overcome problems of dye leakage and photobleaching in time course experiments with rhod-2, single excitation/single emission imaging analyses were conducted with a number of corrective procedures as described earlier (12). First, the fluorescence intensity of rhod-2 was measured in small sampling grids (~4 µm²) in the lamellipodia of well-spread cells to avoid the inclusion of nucleoli, which stain brightly with rhod-2. These sampling grids were sufficiently large that mitochondrial movement in and out of the sampling grid did not materially affect measurements over the time courses we used here. Second, background fluorescence for each set of experiments was obtained in cells that were not incubated with rhod-2 and the background signal for the sampling grid was subtracted from all measurements made in the particular experiment. Adjustments for time-dependent photobleaching (12) were made for each measurement by calculating the bleach-induced rate constant (i.e., time-dependent loss of fluorescence) in untreated cell samples. Third, for verification of the appropriate spatial localization of the rhod-2 staining and to ensure that rhod-2 was not sequestered in lysosomes (32), in some experiments, fibroblasts were coloaded with 100 nM MitoTracker Green and 4.5 µM rhod-2/AM in 0.005% (v/v) Pluronic F-127 for 30 min to determine extent of colocalization. The cells were washed twice and incubated in calcium buffer before imaging. The fluorescence of rhod-2 (Fₚₐ₉) was expressed as arbitrary units. The fluorescence of rhod-2 was not calibrated in terms of absolute [Ca²⁺], because it is not a ratiometric dye.

Mitochondrial membrane potential

Mitochondrial membrane potential (Ψₘ) was measured with JC-1, a fluorescent dye that is concentrated in respiring mitochondria. Cells were incubated with JC-1 (10 µg/ml) for 10 min and washed. Fluorescence measurements were obtained at 1, 2, 3, 5, 10, 15, 20, 25, and 30 min after adding IL-1 using 525/10 excitation and 590/10 emission filters for red J-aggregate fluorescence (Ψₘ sensitive) and 485/10 excitation and 530/10 emission filters for green fluorescence (Ψₘ-insensitive). After subtraction of background values obtained from wells containing JC-1 but devoid of cells, red/green fluorescence ratios were calculated to estimate Ψₘ.
Data analysis

Means and standard errors of the means (SE) were calculated for \([\text{Ca}^{2+}]_i\) measurements, including baseline \([\text{Ca}^{2+}]_i\) and net change in \([\text{Ca}^{2+}]_i\) above baseline. For continuous variables, means and standard errors of the mean were computed and, when appropriate, comparisons between two groups were made with the unpaired Student’s \(t\)-test or with ANOVA for multiple samples. Statistical significance was set at \(P < 0.05\). Experiments were repeated at least 3 times and for each experiment, \(n \geq 3\) replicates were used.

RESULTS

Mitochondria impact IL-1-induced \(\text{Ca}^{2+}\) signals and ERK activation

IL-1 rapidly induced large amplitude, prolonged elevations of \([\text{Ca}^{2+}]_i\) in human gingival fibroblasts on fibronectin-coated substrates (Fig. 1A). However, if mitochondria were depolarized by exposure to either a mixture of antimycin A (5 \(\mu\)g/ml) plus oligomycin (0.5 \(\mu\)g/ml) to inhibit complex III of the respiratory chain and the mitochondrial FoF1 ATP synthase, respectively, or the protonophore FCCP (5 \(\mu\)M), which collapses the proton motive force across the inner mitochondrial membrane, there was a dramatic reduction of the amplitude of IL-1-induced \(\text{Ca}^{2+}\) signals (Fig. 1A). These results were not because of cell death since treatment with ionomycin produced large increases of \([\text{Ca}^{2+}]_i\), similar to untreated cells (Fig. 1A, insets). Accordingly, mitochondria may be important for shaping IL-1-induced \(\text{Ca}^{2+}\) signals (27).

We determined whether mitochondria regulate \(\text{Ca}^{2+}\)-dependent, downstream signals generated by IL-1. ERK is an important, \(\text{Ca}^{2+}\)-dependent MAP kinase in the IL-1 signaling pathway. We have previously demonstrated that increased \([\text{Ca}^{2+}]_i\) is associated with IL-1-induced ERK activation (19), but the mechanism by which calcium handling in discrete stores regulates ERK activation is not defined. As a control, IL-1-induced ERK activity was blocked by plating cells on poly-L-lysine instead of fibronectin (Fig. 1B; Ref. 18). In cells plated on fibronectin and pretreated with antimycin A plus oligomycin or FCCP, IL-1-induced ERK activation was also blocked (Fig. 1C), indicating that mitochondrial function is required for activity. The requirement for mitochondrial function could be bypassed by the calcium ionophore ionomycin, which restored ERK activation after pretreatment of cells with antimycin A/oligomycin or FCCP (Fig. 1D). This observation also excluded the possibility that the inhibition of ERK activation was due to insufficient cytosolic ATP. We also examined the requirement for mitochondrial function in the context of other IL-1-induced signals. Pretreatment of cells with FCCP did not affect IL-1-induced phosphorylation of JNK (Fig. 1E), indicating specific requirements for mitochondrial function in the IL-1 signaling pathway. Collectively, these data indicated that mitochondria play an important and specific role in the generation of IL-1-induced, \(\text{Ca}^{2+}\)-dependent signals.

IL-1-induced mitochondrial \(\text{Ca}^{2+}\) flux

To gain insights into the roles(s) of mitochondrial in cellular \(\text{Ca}^{2+}\) homeostasis, initially we determined the effect of IL-1 treatment on intramitochondrial \(\text{Ca}^{2+}\). In view of the potential artifacts associated with measurement of mitochondrial calcium with compartmentalized rhod-2 (32), we first verified that rhod-2 was indeed associated with mitochondria in human gingival fibroblasts by confocal microscopy. Double labeling with rhod-2 (Fig. 2A, panel 1) and
MitoTracker Green, a mitochondria-specific dye (panel 2), demonstrated that rhod-2 fluorescence and MitoTracker Green fluorescence were largely coincident (panel 3), particularly in lamellipodia where measurements were made, indicating that rhod-2 was largely reporting changes of calcium in the mitochondria and not in endosomes, the ER, or the cytoplasm. Comparison of rhod-2-loaded cells after addition of FCCP (panel 4), showed that FCCP caused loss of mitochondrial-specific rhod-2 fluorescence.

We next verified that antimycin A/oligomycin or FCCP, as described above, could be used to reliably depolarize mitochondria in situ using the \( \Psi_m \)-sensitive dye JC-1. Cells were treated with antimycin A (5 µg/ml) plus oligomycin (0.5 µg/ml) or FCCP (5 µM) or IL-1 (20 ng/ml) for comparison. Cells were then stained with JC-1. Untreated control cells and IL-1-treated cells exhibited numerous, brightly staining mitochondria that emitted orange-red fluorescence. Antimycin A plus oligomycin or FCCP dramatically reduced JC-1 fluorescence (data not shown). The effect of IL-1, antimycin A plus oligomycin or FCCP on mitochondrial membrane potential was estimated from the ratio of red/green JC-1 fluorescence (Fig. 2B). Within 5 min of exposure to antimycin A plus oligomycin or FCCP, the red/green ratio declined by fourfold and remained low thereafter. Treatment with IL-1 only slightly reduced the JC-1 ratio (~10%).

As we established that rhod-2 reflects \([Ca^{2+}]_m\), we determined the effect of IL-1 on \([Ca^{2+}]_m\). Fluorescence signals (F_{rhod2}) are reported as fluorescence arbitrary units (fau). Control cells exhibited a resting \([Ca^{2+}]_m = 20 \pm 5 \) (fau; \( n=5 \)) and, when stimulated with IL-1 (20 ng/ml), demonstrated a 3.5-fold increase of peak \([Ca^{2+}]_m \) (\( n=5 \); \( P < 0.01 \); Fig. 2C; summary data-D). There was no significant \([Ca^{2+}]_m \) increase above baseline in response to IL-1 in cells pretreated with antimycin A (5 µg/ml) plus oligomycin (0.5 µg/ml) or FCCP (5 µM; Fig. 2C, D). These results show that IL-1 induces mitochondrial \( Ca^{2+} \) uptake, which is completely abolished when the membrane potential is dissipated, as expected.

**IL-1 induces mitochondrial ATP production**

We next determined whether IL-1, presumably via mitochondrial \( Ca^{2+} \) uptake, affects cellular ATP production. IL-1 promoted marked increases in cellular ATP levels (Table 1; \( P<0.05 \)). As expected, cells that were pretreated with either antimycin A/oligomycin or with FCCP exhibited substantial (60%) reduction of ATP content. However, even this reduced ATP level was sufficient for the normal function of protein kinases (Fig. 1), as well as for ATP-dependent \( Ca^{2+} \) uptake into ER stores (see below). These data indicated that IL-1 induces increased mitochondrial ATP production, consistent with the \( Ca^{2+} \)-dependent activation of key metabolic enzymes in the intramitochondrial matrix.

**Mitochondria are required for efficient \( Ca^{2+} \) release from the ER in fibroblasts**

The initial increase of \([Ca^{2+}]_i \), after IL-1 stimulation was attributable in part to release of \( Ca^{2+} \) from ER stores as cells loaded with mag-fura-2 showed a very rapid drop of \([Ca^{2+}]_{ER} \) followed by a quick return to baseline (Fig. 3A). Notably, this response was blocked by pretreatment with antimycin A plus oligomycin or FCCP, indicating that IL-1-induced \( Ca^{2+} \) release from ER stores is nearly abolished by mitochondrial depolarization.
We determined whether the dramatic effect of mitochondrial inhibition on Ca\textsuperscript{2+} release from the ER was specific for IL-1 or is seen also with other agonists acting through IP\textsubscript{3}. We measured [Ca\textsuperscript{2+}], after stimulating control cells in Ca\textsuperscript{2+}-free buffer carbachol or PGE\textsubscript{2} and compared these data to [Ca\textsuperscript{2+}], in cells previously exposed to antimycin A and oligomycin. In control cells, carbachol or PGE\textsubscript{2} treatments evoked a large transient increase, indicative of Ca\textsuperscript{2+} release from internal stores. However, if mitochondria were depolarized by pretreatment with antimycin A plus oligomycin (Fig. 3B) or with FCCP (data not shown), the agonist-induced increase of [Ca\textsuperscript{2+}], was reduced by threefold (albeit not completely abolished), indicating that in human gingival fibroblasts, mitochondria are required for Ca\textsuperscript{2+} release from InsP\textsubscript{3}-sensitive stores. These data show that in fibroblasts, mitochondria are key determinants of Ca\textsuperscript{2+} release from internal stores, in contrast to rat basophilic leukemia cells in which no inhibition of carbachol-induced release of Ca\textsuperscript{2+} occurs after mitochondrial depolarization (29).

We assessed whether, primarily, the agonist-induced efflux was suppressed, or whether mitochondria are also essential for allowing Ca\textsuperscript{2+} efflux from the ER through the passive leak pathway. The impact of mitochondrial function on thapsigargin-sensitive Ca\textsuperscript{2+} release was determined in cells with functional mitochondria. Thapsigargin induced a large, transient increase of [Ca\textsuperscript{2+}], above basal levels (Fig. 3C; left panel). In the absence of external Ca\textsuperscript{2+}, if ionomycin was added after the thapsigargin-induced transient, there were only marginal elevations of [Ca\textsuperscript{2+}], suggesting that the ER stores had indeed been emptied by thapsigargin. In sharp contrast, if cells were pretreated with FCCP, thapsigargin induced only a very small increase of [Ca\textsuperscript{2+}], whereas subsequent treatment with ionomycin provoked a well-defined rise (Fig. 3C; right panel). These findings suggested that in the absence of mitochondrial function, even thapsigargin-induced Ca\textsuperscript{2+} release from the ER was impaired. To substantiate this notion, we monitored [Ca\textsuperscript{2+}]\textsubscript{ER}, using ratio fluorimetry in mag-fura-2-loaded cells (31). In cells with functional mitochondria, thapsigargin induced rapid, large-amplitude reductions in the mag-fura-2 ratio. There was almost no response following subsequent treatment with ionomycin, indicating that ER calcium stores were indeed depleted (Fig. 3D; left panel). If, on the other hand, cells were pretreated with FCCP, then thapsigargin was unable to reduce [Ca\textsuperscript{2+}]\textsubscript{ER} substantially, while subsequent treatment with ionomycin reduced [Ca\textsuperscript{2+}]\textsubscript{ER} (Fig. 3D; right panel). This latter observation, when taken together with the similarity of the initial mag-fura-2 ratios in control and FCCP-treated cells, indicated that under these experimental conditions, the reduced ATP levels did not lead to significant reduction in intra-ER calcium. Consequently, grossly reduced ER efflux after mitochondrial inhibition cannot be accounted for by initial metabolic store depletion. Collectively, these data show that independent of the type of stimulus, mitochondria are crucial for Ca\textsuperscript{2+} release from ER stores in human gingival fibroblasts.

We verified the functional coupling between ER stores and mitochondria by investigating the relationship between Ca\textsuperscript{2+} release from the ER and mitochondrial Ca\textsuperscript{2+} uptake. In control cells, thapsigargin (or cyclopiazonic acid, data not shown) induced a ~4-fold increase of [Ca\textsuperscript{2+}]\textsubscript{m} above basal levels (Fig. 3E, left panel; P < 0.01; n=4). Pretreatment of cells with antimycin A/oligomycin or FCCP completely abrogated thapsigargin-induced [Ca\textsuperscript{2+}]\textsubscript{m} transients (Fig. 3E, middle panels; n=4; summary data) as expected, indicating that Ca\textsuperscript{2+} release from the ER is coupled to mitochondrial Ca\textsuperscript{2+} uptake.

We next examined if depolarization of mitochondria by antimycin A and oligomycin or by FCCP blocked IL-1-induced Ca\textsuperscript{2+} release primarily as a result of inhibition of mitochondrial-mediated
Ca\(^{2+}\) uptake or by some other mechanism(s) (e.g., by ATP reduction). To distinguish between these possibilities, we used weak Ca\(^{2+}\) buffering by BAPTA/AM, which can replicate mitochondrial Ca\(^{2+}\) uptake (34) but does not affect the metabolic consequences of mitochondrial inhibition. In cells treated with 5 \(\mu\)M BAPTA/AM, IL-1-induced Ca\(^{2+}\) release was completely blocked, probably because the stores were depleted (Fig. 4A, inset). In contrast, weak buffering (with 1 \(\mu\)M BAPTA/AM) permitted relatively normal release of Ca\(^{2+}\) after IL-1 treatment (Fig. 4A). Accordingly, with this protocol, we assessed the effect of mitochondrial depolarization on Ca\(^{2+}\) release from the ER, in the presence or absence of weak buffering by BAPTA/AM. With this protocol, as before, antimycin/oligomycin or FCCP treatments blocked IL-1-induced Ca\(^{2+}\) release from the ER (Fig. 4B, C). In contrast, replication of Ca\(^{2+}\) buffering by preincubation with BAPTA/AM (1 \(\mu\)M) restored IL-1-induced Ca\(^{2+}\) release from the ER, albeit with somewhat slower kinetics. Thus, in gingival fibroblasts, functional mitochondria are required for mediating IL-1-induced release of Ca\(^{2+}\) from the ER, and this effect appears to be mediated predominantly by the Ca\(^{2+}\) buffering action of mitochondrial Ca\(^{2+}\) uptake.

**IL-1 induces mitochondrial Ca\(^{2+}\) uptake also from non-ER calcium stores**

During the course of these experiments, we noted that a component of the IL-1-induced mitochondrial Ca\(^{2+}\) uptake might originate from a thapsigargin-insensitive (i.e., non-ER) Ca\(^{2+}\) store. Notably, when cells loaded with fura-2 were pretreated with thapsigargin (1 \(\mu\)M; 5 min) in Ca\(^{2+}\)-free buffer, the subsequent addition of IL-1 (20 ng/ml) induced a slow temporal increase of \([\text{Ca}^{2+}]_i\) above baseline levels (Fig. 5A; left panel). This increase of \([\text{Ca}^{2+}]_i\) was not from the ER as cells loaded with mag-fura-2 showed no substantial change in the mag-fura-2 ratio after IL-1 treatment and subsequent treatment with ionomycin only slightly reduced \([\text{Ca}^{2+}]_\text{ER}\) (Fig. 5A; right panel), indicating that the ER was largely depleted of Ca\(^{2+}\).

We also repeated these experiments using 2-APB instead of thapsigargin to inhibit IP\(_3\) receptors on the ER. We noted a similar, slow increase of \([\text{Ca}^{2+}]_i\), after IL-1 treatment and no significant reduction of the mag-fura-2 ratio (Fig. 5B; left panel). However, there was a dramatic reduction of \([\text{Ca}^{2+}]_\text{ER}\) after subsequent treatment with ionomycin (Fig. 5B; right panel), indicating that the stores were still full. Thus, IL-1 can slowly release Ca\(^{2+}\) from thapsigargin-insensitive stores that do not correspond functionally to ER stores. Importantly, in the presence of thapsigargin or 2-APB, IL-1 still promoted pronounced increases of \([\text{Ca}^{2+}]_m\) in rhod-2-loaded cells, which were completely blocked by FCCP (Fig. 5C, D). Thus, after IL-1 treatment, mitochondria accumulate Ca\(^{2+}\) released both from the ER and intracellular non-ER stores.

**Mitochondria regulate Ca\(^{2+}\) entry through store-operated channels**

In addition to the ER, the other major source of the cytosolic Ca\(^{2+}\) signal is a Ca\(^{2+}\) influx through the plasma membrane via store-operated channels (SOCs). As we found that mitochondria are essential for normal store emptying in fibroblast, it was clear that mitochondria are also required for normal SOC function through this indirect mechanism. However, we sought to determine whether mitochondria have a more direct impact on SOC function per se as has been reported for rat basophilic leukemia cells earlier (29). To address this question, the intracellular Ca\(^{2+}\) stores were first depleted in fura-2-loaded fibroblasts by thapsigargin in a Ca\(^{2+}\)-free buffer, and then the mitochondrial membrane potential was either preserved or was dissipated with oligomycin/antimycin A or FCCP. SOC function was then assessed by monitoring the effect of
addition of external Ca$^{2+}$ on [Ca$^{2+}$]$_i$. External Ca$^{2+}$ added to cells with intact mitochondria caused a rapid increase of cytosolic Ca$^{2+}$, which then decayed slowly (Fig. 6A). However, when mitochondria were depolarized after store depletion, subsequent readmission of Ca$^{2+}$ generated a smaller-amplitude Ca$^{2+}$ signal, which increased less steeply (Fig. 6A; $n=4$). We measured the initial rate of rise of the Ca$^{2+}$ signal (initial slope) as this parameter better correlates with the Ca$^{2+}$ flux of open store-operated channels than the amplitude of the peak signal (29). The slope was significantly reduced by either antimycin A plus oligomycin or by FCCP compared with control recordings from the same cell preparations ($P<0.05$; Fig. 6A; right panel; $n=4$). Thus mitochondria promote Ca$^{2+}$ entry through store-operated channels, and this effect may predominate over the [Ca$^{2+}$]-reducing effect of mitochondrial Ca$^{2+}$ uptake.

Next, we assessed the overall (direct and indirect) impact of mitochondria on SOC activity under conditions when SOC was stimulated by the natural agonist IL-1. To monitor SOC, we used Mn$^{2+}$-quenching of fura-2 fluorescence in IL-1-treated cells. This method is superior to conventional analyses of Ca$^{2+}$ entry because the only source of Mn$^{2+}$ is the extracellular space, and Mn$^{2+}$ is not removed from the cytosol by Ca$^{2+}$ pumps. As expected, control cells showed marked quenching of fura-2 fluorescence after addition of IL-1, indicating cation entry via the plasma membrane. In contrast, cells pretreated with either antimycin A/oligomycin or FCCP exhibited much less quenching (Fig. 6B). We also performed the reverse experiment in which cells were pretreated with IL-1 and Mn$^{2+}$-containing buffer was then added (Fig. 6C). These results were in complete agreement with the first set of quenching experiments and indicate that mitochondria play a crucial role in regulating store-operated Ca$^{2+}$ influx.

**DISCUSSION**

Our major finding is that IL-1-induced ERK activation is dependent on the ability of mitochondria to regulate IL-1-induced Ca$^{2+}$ signals. As IL-1-induced activation of ERK is a critical step in inflammatory destruction of extracellular matrices through the expression of matrix metalloproteinases (7, 8), mitochondrial dysfunction in inflammation and sepsis may be an important determinant of the duration and amplitude of inflammatory connective tissue diseases through its regulation of IL-1-induced calcium signals. Although previous work has established a physiological relationship between calcium regulation and mitochondria (27–30, 33–35), the pathophysiological consequences of mitochondrial dysfunction in inflammatory signaling have not been defined. Here, we demonstrate that the impact of mitochondria on calcium signaling is important for appropriate cytokine regulation of cell function in inflammation. Moreover, under inflammatory conditions, mitochondrial functions can be impaired (9), which, in turn, could seriously alter the extent and kinetics of tissue destruction. Our data suggest that in inflammatory environments, reduced mitochondrial respiration, and calcium handling activity could impact significantly on the duration and amplitude of IL-1-dependent signals, important regulators of connective tissue metabolism and tissue remodeling (1).

Mitochondrial control of local calcium signaling and localization of effectors close to the source of the calcium signal may provide a common mechanism that underlies activation of mitochondrial uptake sites (33, 35). In view of the considerable heterogeneity of mitochondrial function both within individual cell types and between different cell types (36), it is not surprising that different cell types exhibit variable responses to agonists, including IL-1.
Remarkably, in the absence of normal mitochondrial Ca\(^{2+}\) handling, the IL-1 induced Ca\(^{2+}\) signal is almost completely blunted in gingival fibroblasts, which points to a uniquely important role for mitochondria in the signaling process induced by a key inflammatory cytokine. Indeed, the extraordinary versatility of the various components of the Ca\(^{2+}\)-signaling machinery enables dramatically different roles for mitochondria in generating the global Ca\(^{2+}\) signal, depending on the particular stimulus and the target tissue. For example, mitochondrial depolarization does not affect carbachol-induced release of Ca\(^{2+}\) from the ER in rat basophilic leukemia cells (29) while in bradykinin-stimulated BHK cells, mitochondria promote agonist-induced release of Ca\(^{2+}\) from the ER (34). Although these latter data most closely approximate our observations in IL-1-stimulated fibroblasts, the effects of bradykinin on BHK cells were much less dramatic than the gingival fibroblasts examined here. These differences may be due to the variation of spatial organization of mitochondria in fibroblasts, many of which are closely related to the endoplasmic reticulum and the cell membrane. Accordingly, this proximity may facilitate mitochondrial regulation of ER calcium stores. In addition, the wide range of these apparently contradicting effects may be explained on Ca\(^{2+}\)-dependence of ER efflux.

In most cellular systems, IP\(_3\)-receptor-mediated Ca\(^{2+}\) release exhibits a bell-shaped dependence on extra-ER Ca\(^{2+}\): although a slight increase in Ca\(^{2+}\) at the external side of the receptor facilitates Ca\(^{2+}\) release and potentiates IP\(_3\) sensitivity, further elevation is strongly inhibitory. Accordingly, the direction and magnitude of the global effect of mitochondrial Ca\(^{2+}\) uptake on ER release will depend on a variety of factors, including the number and Ca\(^{2+}\) sensitivity of the stimulated IP\(_3\) receptors, the on- and off-rates of the facilitating and inhibitory Ca\(^{2+}\) binding sites, as well as the number and spatial organization of mitochondria near the ER (27–29, 35; and see above). If mitochondrial Ca\(^{2+}\) suppresses the near-ER Ca\(^{2+}\) below the optimum, mitochondrial inhibition will facilitate release from the store. If, however, an initially small Ca\(^{2+}\) release from the ER generates sufficiently high localized [Ca\(^{2+}\)] to inhibit further release, continuous mitochondrial Ca\(^{2+}\) uptake will be required to enable optimal Ca\(^{2+}\) efflux from the store, and therefore, the inhibition of the mitochondrial Ca\(^{2+}\) uptake will interfere with normal ER release. Our data show that in IL-1-stimulated fibroblasts, mitochondria are required for ER release, and we suspect that the major reason for this is the proximity of mitochondria to the ER, particularly in the vicinity of focal adhesions.

Several arguments support the notion that the dissipation of the mitochondrial membrane potential affects ER release primarily because of the cessation of the mitochondrial Ca\(^{2+}\) uptake and not due to other alterations (e.g., loss of cellular ATP or interference with Ca\(^{2+}\)-releasing second messengers). Specifically, mitochondrial inhibition caused no major change in the Ca\(^{2+}\) within the ER as verified by measurements of the mag-fura-2 ratio and the magnitude of the ionomycin-releasable ER pool. More importantly, a modest chelation of cytosolic Ca\(^{2+}\) by BAPTA, a maneuver that mimics mitochondrial Ca\(^{2+}\) buffering but fails to influence the metabolic consequence of mitochondrial inhibition (33), largely restored the IL-induced Ca\(^{2+}\) release from the ER. This finding clearly shows that in cells with impaired mitochondrial function, IL-1 was still able to generate Ca\(^{2+}\)-releasing second messengers (presumably IP\(_3\)), and the reduction in cellular ATP was not responsible for the dramatic inhibition of store emptying. Notably, we also found that interference with mitochondrial functions strongly inhibits thapsigargin-induced release as well. Although a small percentage of IP\(_3\) receptors may be functional under resting conditions, the generally accepted concept is that the majority of the thapsigargin-induced efflux occurs through a separate route, a passive leak pathway (37, 38).
Since ATP was reported to facilitate the leak pathway (37), it is conceivable that reduction of ATP after mitochondrial depolarization might contribute to the reduced thapsigargin-induced efflux. Alternatively, the leak pathway may also be inhibited by near-ER Ca\textsuperscript{2+}, similar to the specific release channels.

We have found that mitochondrial calcium buffering in IL-1-induced calcium signaling affects not only the initial release of calcium from the ER but also the subsequent activation of store-operated channels, as has been shown for the IP\textsubscript{3} releasing agonist carbachol in rat basophilic leukemia cells (29). Previous reports of these cells showed that a calcium-dependent, slow inactivation of calcium influx is regulated by mitochondrial buffering of cytosolic calcium (27). We found that the absence of this buffering in cells with depolarized mitochondria also blocks IL-1-induced activation of ERK, which we bypassed with ionomycin, an experimental system for promoting prolonged calcium entry. Prolonged calcium elevation, which is typically seen after treatment with IL-1, is thus apparently an important prerequisite for ERK activation and underlines the importance of mitochondria in shaping the global cytosolic calcium signals that are generated by IL-1. Previous reports (14, 15, 39, 40) have emphasized the importance of calcium signaling in gene expression, and our results here not only provide a specific pathophysiological example but also show that the temporal dynamics of the IL-1-induced calcium signal is regulated by mitochondria. As experimentally demonstrated by the bypass experiments with ionomycin, these calcium signals are, in turn, critical for generation of an important downstream signal (i.e., ERK activation).

ACKNOWLEDGMENTS

The research was funded by an operating grant from the CIHR to G.P.D. and C.A.M., a Group Grant to C.A.M. and the Canadian Arthritis Network. G.P.D. holds the R. Fraser Elliott Chair in transplantation research from the Toronto General Hospital of the University Health Network and a Tier 1 Canada Research Chair.

REFERENCES


Received Aug. 3, 2004; accepted December 31, 2004.
**Table 1**

ATP Levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATP (pmol/cell/min)</th>
<th>Increase (+) or decrease (−) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.66 ± 0.087</td>
<td>—</td>
</tr>
<tr>
<td>IL-1</td>
<td>3.19 ± 0.18</td>
<td>+92.17*</td>
</tr>
<tr>
<td>Antimycin A/Oligomycin</td>
<td>0.61 ± 0.029</td>
<td>−63.25*</td>
</tr>
<tr>
<td>FCCP-pretreatment</td>
<td>0.65 ± 0.027</td>
<td>−60.84*</td>
</tr>
</tbody>
</table>

FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone. Values are means ± SD of five replicates and ATP content (pmol/cell/min) were determined. *P < 0.05.
**Figure 1. Mitochondria impact IL-1-induced ERK activation.**

**A)** Fibronectin-attached cells in calcium buffer were pretreated with antimycin A (5 µg/ml) plus oligomycin (0.5 µg/ml) or FCCP (5 µM) for 20 min and then incubated with IL-1 (20 ng/ml). [Ca^{2+}]_i was measured in fura-2 loaded cells. Insets show single cells pretreated with either antimycin/oligomycin or FCCP and then exposed to ionomycin (2 µM) to establish cell viability and maintenance of intact calcium stores.

**B)** Fibroblasts plated on fibronectin (FN) or poly-L-lysine (PL; to block IL-1-induced calcium release) were either untreated or incubated with IL-1 as indicated. Phospho-ERK activity was assessed by separating lysates with SDS-PAGE and probing blots for phospho-ERK. Total ERK was assessed by stripping and reprobing blots with rabbit polyclonal anti-ERK.

**C)** Cells on fibronectin were preincubated with antimycin A (5 µg/ml) plus oligomycin (0.5 µg/ml) and 5 µM FCCP for 20 min before addition of IL-1 (20 ng/ml). Control cells (C) were not pretreated.

**D)** ERK activation was restored after previous mitochondrial depolarization with antimycin A/oligomycin or FCCP if cells were treated simultaneously with ionomycin (2 µM) in addition to IL-1 (20 ng/ml) for 15 min.
**Figure 2. IL-1-induced mitochondrial Ca\(^{2+}\) flux.**

**A** Colocalization of calcium indicator rhod-2 (panel 1) and MitoTracker Green (panel 2) in cells plated on fibronectin. Merge of two images (panel 3) demonstrates colocalization of two dyes in stained mitochondria. Panel 4 shows distribution and weak staining of rhod-2-stained mitochondria after dissipation of mitochondrial membrane potential with FCCP (5 µM).

**B** Time course measurements of mitochondrial membrane potential (\(\Psi_m\)) measured by ratio of red/green JC-1 fluorescence in cells treated with IL-1 (20 ng/ml), antimycin A (5 µg/ml) plus oligomycin (0.5 µg/ml) or FCCP (5 µM) at indicated times after treatment. Mitochondrial calcium ([Ca\(^{2+}\)]\(_m\)) was estimated by rhod-2 fluorescence after adjustment for photobleaching and dye leakage as described under Materials and Methods. Fluorescence signals were obtained from extranuclear regions of interest. \(F_{\text{rhod2}}\) data are shown as fluorescence arbitrary units (fau). Cells plated on fibronectin were loaded with rhod-2 and treated with IL-1. Cells grown on fibronectin were pretreated with antimycin A (5 µg/ml) plus oligomycin (0.5 µg/ml; Antimycin A/oligo-pretreatment) or FCCP (5 µM) for 20 min before IL-1. Graphs show corresponding traces of [Ca\(^{2+}\)]\(_m\) expressed as means ± SE fluorescence arbitrary units from at least 5 cells per time point.

**D** Summary data (means ± SE) from \(n = 4–5\) experiments. Data show estimated maximum [Ca\(^{2+}\)]\(_m\) above baseline in cells plated on fibronectin and either treated with IL-1 alone (control), pretreated with antimycin A plus oligomycin (Anti. A/Oligo), or FCCP (FCCP) for 20 min followed by IL-1. Mitochondrial depolarization significantly reduced [Ca\(^{2+}\)]\(_m\) compared with controls (\(P < 0.001\)).
Figure 3. Mitochondria are required for efficient Ca\textsuperscript{2+} release from the endoplasmic reticulum. A) Measurement of [Ca\textsuperscript{2+}]_ER by mag-fura-2 ratio fluorescence shows that IL-1 induces rapid loss of the mag-fura-2 ratio, which is completely blocked by mitochondrial depolarization using antimycin/oligomycin or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) treatments using the same depolarization protocols as in Fig. 2. B) Whole cell calcium measurements [Ca\textsuperscript{2+}] in fura-2-loaded cells were obtained in calcium-free medium plated on fibronectin. Carbachol (300 nM) or PGE\textsubscript{2} (1 \mu M) treatments promote calcium release from intracellular stores and this release is reduced >fivefold by mitochondrial depolarization with antimycin/oligomycin. C) [Ca\textsuperscript{2+}] measurements in fura-2-loaded cells in calcium-free medium show sharp increases after thapsigargin (TG; 1 \mu M) treatments and little subsequent increase after ionomycin (2 \mu M). Mitochondrial depolarization by FCCP (5 \mu M; 20 min.) largely blocks thapsigargin-induced calcium increase (left panel), but ionomycin causes a prolonged increase of [Ca\textsuperscript{2+}]_ER, (right panel). D) Similar experimental design as C, but cells were loaded with mag-fura-2 for estimates of [Ca\textsuperscript{2+}]_ER. In control cells thapsigargin induces a sharp and rapid decrease of the mag-fura-2 ratio that is not affected later by ionomycin (2 \mu M). In contrast, after mitochondrial depolarization with FCCP (20 min), thapsigargin causes only a small and prolonged decrease of the mag-fura-2 ratio, which is strongly enhanced by subsequent treatment with ionomycin. All data are representative of three separate experiments. E) Representative [Ca\textsuperscript{2+}]_im traces of rhod-2 loaded cells plated on fibronectin, pretreated with antimycin A plus oligomycin (Antimycin A/Oligomycin pretreatment) or FCCP (FCCP pretreatment), and then treated with thapsigargin (1 \mu M). [Ca\textsuperscript{2+}]_im was measured as described in Experimental Procedures. Each trace shows means ± SE of 4–5 independent samples. Aggregate data in histogram are from n = 4–5 experiments. Mitochondrial depolarization significantly inhibited IL-1-induced [Ca\textsuperscript{2+}]_im increases above baseline levels (P<0.001).
Figure 4. Mitochondrial calcium uptake is required for IL-1-induced calcium release from the endoplasmic reticulum. A) Cells on fibronectin loaded with mag-fura-2 show sharp reduction of mag-fura-2 ratio after IL-1 treatment (left panel), indicating release of calcium from the endoplasmic reticulum (ER). If cytoplasm is subjected to weak calcium buffering by BAPTA/AM (1 µM), IL-1-induced reduction of mag-fura ratio is preserved. If stronger calcium buffering is used (5 µM BAPTA/AM), IL-1 induces only limited calcium release from the ER. B, C) Pretreatment (20 min) with antimycin/oligomycin (B) or FCCP (C) blocks IL-1-induced release of calcium from the ER. If weak calcium buffer is used (1 µM BAPTA/AM pretreatment), the IL-1-induced calcium release is largely preserved. These individual traces are representative of 5 separate experiments for each condition.
Figure 5. IL-1 induces mitochondrial Ca\(^{2+}\) uptake from other, non-ER internal calcium stores. 

A) Cells plated on fibronectin preincubated with thapsigargin (1 µM; 15 min.) were loaded with fura-2 or mag-fura-2 in calcium-free buffer and then treated with IL-1 (20 ng/ml; *left panels*) or with IL-1 followed by ionomycin (2 µM) to show whether ER stores were full or empty. [Ca\(^{2+}\)]\(_i\), and mag-fura-2 ratio were measured in single cells. The baseline [Ca\(^{2+}\)]\(_i\) is shown as a dotted line. 

B) Same experimental protocol as (A), except cells were preincubated with 2-APB (75 µM) instead of thapsigargin. 

C, D) Measurements of [Ca\(^{2+}\)]\(_{m}\) in cells preincubated with thapsigargin (1 µM; *panels in C*) or 2-APB (75 µM; panels in D), loaded with rhod-2 to estimate [Ca\(^{2+}\)]\(_{m}\) and then stimulated with IL-1. *Panels on right* show cells that were depolarized 20 min before treatments with FCCP. Inset in (D, *left panel*) shows [Ca\(^{2+}\)]\(_{m}\) of 5 cells pretreated with 2-APB followed by IL-1 treatment. Data are means ± SE of 5 different cells and are representative of 3 separate experiments.
**Figure 6. Mitochondrial depolarization reduces rate of Ca\textsuperscript{2+} entry.**

**A** After depleting stores with thapsigargin (1 µM) in fura-2-loaded cells, readdition of external Ca\textsuperscript{2+} (2 mM) in buffer results in rapid increases of [Ca\textsuperscript{2+}]\textsubscript{i} (control). Depolarizing mitochondria with either antimycin A (5 µg/ml) plus oligomycin (0.5 µg/ml) or FCCP (5 µM; both for 20 min) substantially reduces calcium entry. Histogram compares initial slopes of Ca\textsuperscript{2+} signals in control cells after Ca\textsuperscript{2+} readdition with cells pretreated with either antimycin A plus oligomycin or FCCP. Numbers of cells (n) for each condition are control, n = 6; antimycin A plus oligomycin (Anti A/Oligo), n = 5; FCCP, n = 5.

**B** Manganese-quenching experiments of fura-2-loaded cells were performed in buffer containing MnCl\textsubscript{2} (1 mM). Typical traces of photon counts of control and antimycin A plus oligomycin (Anti A/Oligo) or FCCP-pretreated cells. Control cells show sharp reductions of fura-2 fluorescence when excited at 356 nm (isosbestic point) after adding IL-1. Cells pretreated with either antimycin A plus oligomycin or FCCP exhibited much less quenching.

**C** In the reverse experiment, control and antimycin A plus oligomycin (Anti A/Oligo) or FCCP-pretreated cells were incubated with IL-1 (20 ng/ml) and Mn\textsuperscript{2+}-containing buffer were then added.