

Research Article

Close Functional Coupling Between Ca^{2+} Release-Activated Ca^{2+} Channels and Reactive Oxygen Species Production in Murine Macrophages

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Aim. To investigate the role of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in the ROS production in macrophages. **Methods.** The intracellular $[\text{Ca}^{2+}]_i$ was analyzed by confocal laser microscopy. The production of ROS was assayed by flow cytometry. **Results.** Both LPS and thapsigargin induced an increase in intracellular $[\text{Ca}^{2+}]_i$, either in the presence or absence of extracellular Ca^{2+} in murine macrophages. The Ca^{2+} signal was sustained in the presence of external Ca^{2+} and only initiated a mild and transient rise in the absence of external Ca^{2+} . CRAC channel inhibitor 2-APB completely suppressed the Ca^{2+} entry signal evoked by thapsigargin, and suppressed approximately 93% of the Ca^{2+} entry signal evoked by LPS. The increase in intracellular $[\text{Ca}^{2+}]_i$ was associated with increased ROS production, which was completely abolished in the absence of extracellular Ca^{2+} or in the presence of CRAC channel inhibitors 2-APB and Gd^{3+} . The mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone and the inhibitor of the electron transport chain, antimycin, evoked a marked increase in ROS production and completely inhibited thapsigargin and LPS-evoked responses. **Conclusions.** These findings indicate that the LPS-induced intracellular $[\text{Ca}^{2+}]_i$ increase depends on the Ca^{2+} entry through CRAC channels, and close functional coupling between CRAC and ROS production in murine macrophages.

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INTRODUCTION

In nonexcitable cells, one major route for Ca^{2+} influx is through store-operated Ca^{2+} channels (SOC) in the plasma membrane [1]. In some cell types including macrophages, store-operated Ca^{2+} influx channels are also called Ca^{2+} release-activated Ca^{2+} (CRAC) channels [1, 2]. However, the downstream consequences of CRAC channels activation are not clearly established. In Jurkat T cells, Ca^{2+} entry through CRAC channels is required for T-cell receptor-mediated activation of nuclear transcription factors that regulate the expression of cytokine genes central to the immune response [3]. In RBL-1 cells, Ca^{2+} entry through CRAC channels stimulates arachidonic acid production and leukotriene secretion [4]. Dysfunction of CRAC has been linked to severe combined immunodeficiency [5], acute pancreatitis [6], and Alzheimer's disease [7]. CRAC inhibition attenuates neutrophil function and postshock acute lung injury in rats [8].

Professional phagocytes generate high levels of reactive oxygen species (ROS) using a superoxide-generating NADPH oxidase as part of their armory of microbicidal mechanisms, and ROS production is largely dependent on $[\text{Ca}^{2+}]_i$ mobilization [9]. LPS increases intracellular calcium concentration and ROS production in macrophages [10]. There are a number of different Ca^{2+} channel types found in nonexcitable cells, such as macrophages [11]. In Kupffer cells (liver macrophages), LPS causes the irrepressible influx of calcium via L-type voltage-dependent calcium channels [12]. Macrophage activation by a vanadyl-aspirin complex is dependent on L-type calcium channel [13]. Yet, it is not clear whether LPS-induced intracellular Ca^{2+} increase depends on CRAC channels and the relationship between CRAC calcium entry and ROS production in macrophages.

The aim of this study was to use Fluo-3/AM and DCFH-DA as a probe to examine intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) changes and ROS generation in RAW264.7

macrophages challenged with LPS or thapsigargin. Our hypothesis was that LPS-induced intracellular $[Ca^{2+}]_i$ increase depends upon CRAC channels, and CRAC channels are essential for ROS production in murine macrophages.

MATERIALS AND METHODS

Experimental protocol

RAW264.7 cells were treated with LPS (2, 10 $\mu\text{g}/\text{mL}$) (*Escherichia coli* O111: B4, Sigma, USA) or thapsigargin (1, 2 μM) (ALEXIS, USA) in both the presence and absence of external Ca^{2+} . Intracellular calcium was monitored using laser confocal microscopy. ROS were measured by flow cytometry.

Cell culture

RAW264.7 cells (obtained from the China Center for Type Culture Collection, CCTCC, Shanghai, China) were cultured (37°C, 5% CO_2) in RPMI 1640 medium (Gibco, BRL) with 10% fetal bovine serum and penicillin-streptomycin. For Ca^{2+} imaging experiments, cells were passaged onto 35 mm culture dishes containing glass coverslips (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and used 24 to 48 hours after plating.

Intracellular Ca^{2+} measurements

Fluo-3/AM (5 μM) (Biotium, Calif, USA) was added into the incubation medium and incubated for 30 minutes at 37°C. Cells were washed free of extracellular Fluo-3/AM dye in standard external solution containing (in mM) NaCl 145, KCl 2.8, $CaCl_2$ 2, $MgCl_2$ 2, D-glucose 10, and HEPES 10, pH 7.4, with NaOH. Ca^{2+} -free solution was a standard external solution without Ca^{2+} ; the Ca^{2+} was substituted with 1 mM EGTA. Fluorescence measurements of $[Ca^{2+}]_i$ were performed using confocal laser scanning microscopy (Olympus FV500, Japan) with Olympus IX71 camera in the presence and absence of Ca^{2+} in the bath. Fluo-3 was excited at 488 nm and emission measured between 500 and 550 nm. Images of 512×512 pixels were acquired with a $20 \times$ objective. Laser scanning was started to obtain a time series of images. Acquisition rate was 1 frame (512×512) per 15 seconds. The obtained images were quantitatively analyzed for changes in fluorescence intensities within regions of interest (ROIs) using the Olympus FV500 Vision software. In each cell well, at least 15 equivalent-sized ROIs were identified, monitored, and analyzed during the experimental period. Fluorescence intensity and the curve of the time course were analyzed by the computer automatically. Increase in $[Ca^{2+}]_i$ is expressed as a ratio: fluorescence intensity of Fluo-3 over baseline (fluorescence/baseline). This ratio method is used because it is independent of factors such as dye concentration, excitation intensity, and detector efficiency.

The flow cytometric assay of ROS

The dye, DCFH-DA, has been used to measure intracellular generation of ROS. The method is based on the fact

that DCFH-DA diffuses through the cell membrane and it is hydrolyzed by intracellular esterases to DCFH, which remains trapped within the cells. DCFH, a nonfluorescent compound, is able to react with ROS, and to generate the fluorescent 2',7'-dichlorofluorescein (DCF). Thus fluorescence intensity is proportional to the amount of ROS produced by the cells. Briefly, cells were harvested and suspended at a concentration of 1×10^6 cells/mL in PBS. Cells were washed with PBS and incubated with DCFH-DA (2',7'-dichlorofluorescein diacetate, Molecular Probes, Ore, USA) (10 μM) for 20 minutes at 37°C in the dark. After washing twice with cold PBS, cells were analyzed by flow cytometry (Becton-Dickinson, NJ, USA). FL1 amplifier mode, DCFH, was excited at 488 nm and emitted at 525 nm. Phorbol 12-myristate 13-acetate (PMA) 10 μM was used as a positive control. We determined M1 and M2 by mean fluorescence intensity as the gate, data were expressed as % gated, M1 stands for the percentage of negative cells, M2 stands for the percentage of positive cells. Approximately 10 000 cells were analyzed per group.

Statistical analysis

Data are expressed as mean \pm SD. The statistical analysis was carried out using SPSS 11.0 programs (SPSS, Chicago, Ill). All data were analyzed by one-way analysis of variance followed by *Student-Newman-Kuels* post hoc test for multiple comparisons. $P < .05$ was considered the threshold for statistical significance between the control group and the experimental groups.

RESULTS

Thapsigargin-evoked Ca^{2+} influx but not Ca^{2+} release stimulates ROS production

In RAW264.7 cells, the SERCA inhibitor thapsigargin depletes internal Ca^{2+} stores and subsequently activates CRAC channels. The intracellular $[Ca^{2+}]_i$ in RAW264.7 cells was determined fluorometrically with the calcium indicator Fluo-3/AM as described previously. The upper panel in Figure 1(a) depicts RAW264.7 cells preloaded with Fluo-3/AM, and the lower panel shows typical Ca^{2+} responses obtained after stimulation with thapsigargin (2 μM). In the absence of external Ca^{2+} , thapsigargin triggered Ca^{2+} release from the internal stores, and the Ca^{2+} signal decayed back to prestimulation levels over several minutes. In the presence of external Ca^{2+} , however, the Ca^{2+} signal was sustained. Dimethyl sulphoxide (DMSO) used as the thapsigargin vehicle did not produce any significant $[Ca^{2+}]_i$ change (Figure 1(b)). To test the relationship between Ca^{2+} influx through CRAC channels and ROS production, we measured ROS formation at different times after stimulation with thapsigargin in the presence and absence of external Ca^{2+} . The results are shown in Figures 2(a), 2(b). The addition of thapsigargin into the culture medium induced an elevation in ROS at 1 minute, which reached maximal levels within 10 minutes and returned to basal levels in 20 minutes. Thapsigargin stimulated

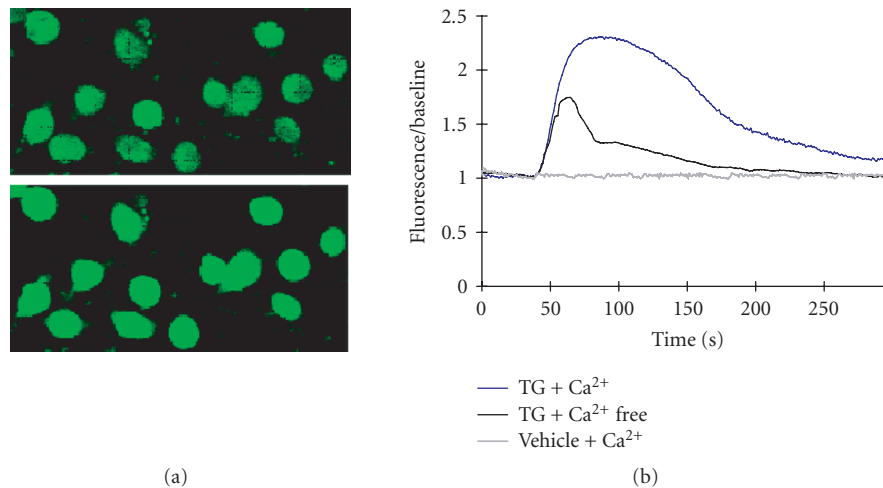


FIGURE 1: Thapsigargin induced an elevation of $[Ca^{2+}]_i$ in RAW264.7 cells. (a) The upper panel is an image of resting RAW264.7 cells loaded with Fluo-3/AM, and the lower panel shows typical Ca^{2+} responses obtained after stimulation with thapsigargin ($2 \mu M$) in the presence of external Ca^{2+} . (b) Intracellular Ca^{2+} signals to thapsigargin in the absence and presence of external Ca^{2+} . Figure 1(b) is representative of four experiments performed on different experimental days. TG: thapsigargin.

ROS production in a dose-dependent manner (Figures 2(c), 2(d)), direct voltage-gated Ca^{2+} channel blockade nifedipine did not suppress the ROS production (Figure 2(d)). No increase in ROS formation could be detected in the 10 minutes following stimulation with thapsigargin in a Ca^{2+} -free solution (Figure 2(a)).

The CRAC channels inhibitors

2-Aminoethoxydiphenylborate (2-APB) and Gd^{3+} block thapsigargin-dependent stimulation of ROS production

$20 \mu M$ 2-APB completely suppressed the Ca^{2+} entry signal evoked by $2 \mu M$ thapsigargin (Figure 3(a)). 2-APB also prevented the ability of thapsigargin to release ROS (Figure 3(b)). Similar findings were obtained with $10 \mu M$ Gd^{3+} (Figure 3(b)).

LPS stimulates Ca^{2+} entry and $[Ca^{2+}]_i$ increase through CRAC channels

The addition of LPS ($2 \mu g/mL$) into the culture medium induced an elevation in $[Ca^{2+}]_i$ which reached maximal levels within 90 seconds and slowly returned to basal levels. However, the Ca^{2+} signal was sustained with $10 \mu g/mL$ LPS (Figure 4(a)). Thapsigargin ($1 \mu M$) produced a slow $[Ca^{2+}]_i$ increase, followed by a return to basal levels. After thapsigargin treatment, the effect of LPS ($10 \mu g/mL$) on $[Ca^{2+}]_i$ increase was completely abolished (Figure 4(b)). The rate of Ca^{2+} entry (measured following readmission of external Ca^{2+}) was significantly slower when CRAC channel blocker 2-APB ($20 \mu M$) was applied (Figures 4(c), 4(d)).

LPS-evoked Ca^{2+} influx stimulates ROS production

The addition of LPS ($10 \mu g/mL$) into the culture medium increased ROS production which reached maximal levels

within 20 minutes and slowly returned to basal levels in 16 hours (Figures 5(a), 5(b)). At 20 minutes, the stimulation of ROS production with LPS ($10 \mu g/mL$) was suppressed in the absence of external Ca^{2+} , or when pretreated with $20 \mu M$ 2-APB (Figures 5(a), 5(b)).

Effect of FCCP and antimycin on thapsigargin and LPS-evoked generation of ROS

To test whether ROS source was located in the mitochondria, we carried out a set of experiments in which macrophages were incubated in the presence of the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) and/or the inhibitor antimycin. When the FCCP ($1 mM$) was added to the cells, a significant increase in ROS production was observed. In the presence of FCCP, stimulation of cells with $2 \mu M$ TG or $10 \mu g/mL$ LPS failed to induce further increases in ROS level (Figure 6(a)).

We further characterized the origin of ROS within the mitochondria, and performed a series of experiments by incubation of macrophages in the presence of the electron transport chain inhibitor antimycin. Treatment of cells with $5 mM$ antimycin led to a significant increase in ROS generation. In the presence of the mitochondrial inhibitor, stimulation of cells with $10 \mu g/mL$ LPS failed to evoke further increases in ROS production. A similar result was obtained when the cells were challenged with TG ($2 \mu M$) in the presence of the inhibitor (Figure 6(b)).

DISCUSSION

The measurement of $[Ca^{2+}]_i$ in RAW264.7 cells has provided evidence that LPS stimulates the entry of extracellular Ca^{2+} . Support for an increased entry of extracellular Ca^{2+} via CRAC channels was obtained from three sources. Firstly,

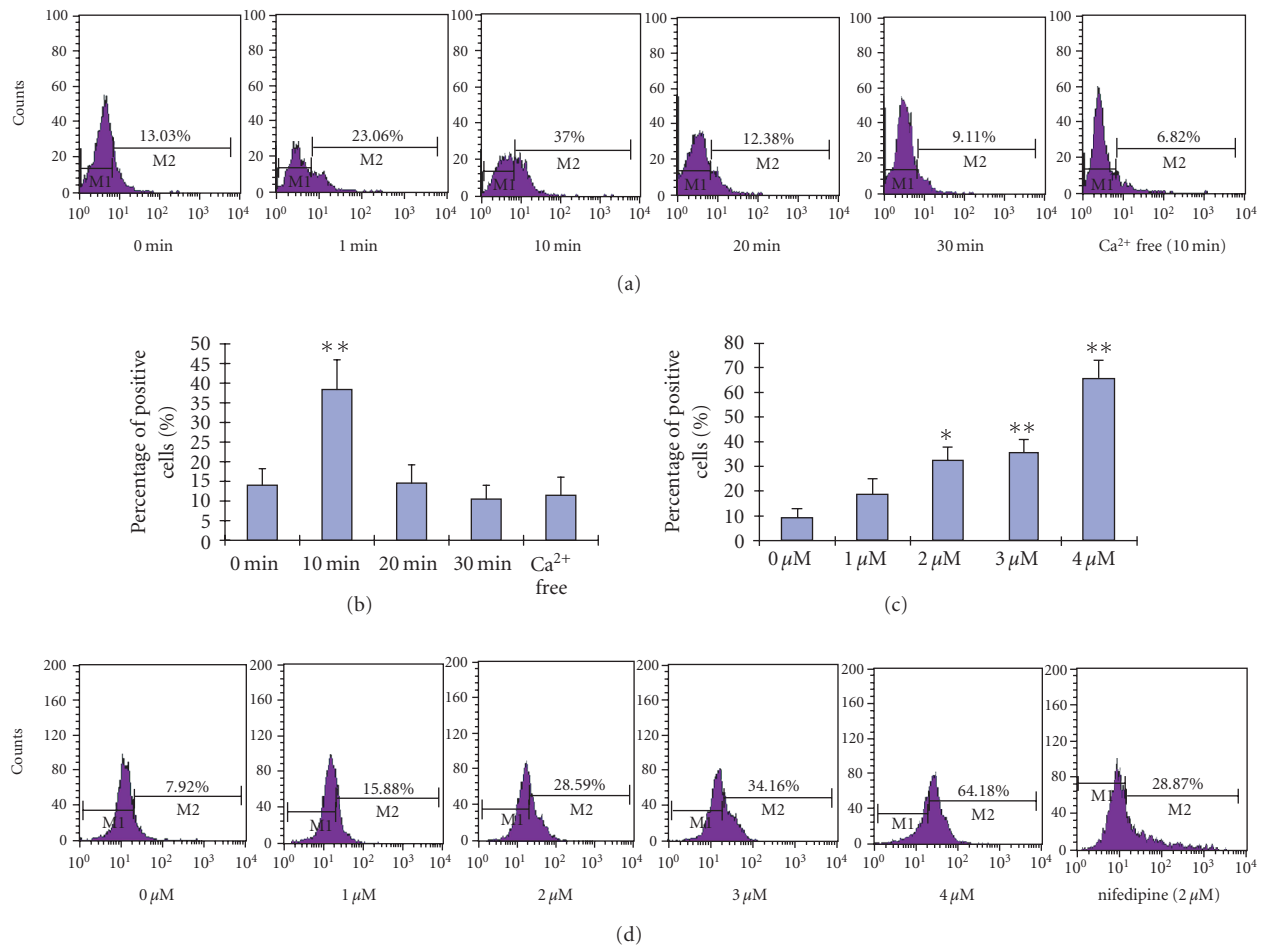


FIGURE 2: Thapsigargin stimulated ROS production. (a), (b) Flow cytometry profiles showing the time course of ROS production following stimulation with thapsigargin. RAW246.7 cells were subjected to thapsigargin (2 μM). ROS were measured by a flow cytometry. ROS production was suppressed in the absence of external Ca²⁺ over 10 minutes. (c), (d) Thapsigargin increased the production of ROS in a dose-dependent manner in the presence of external Ca²⁺ (at 10 minutes). Three independent experiments have been performed. **P* < .05 versus control group; ***P* < .01 versus control group.

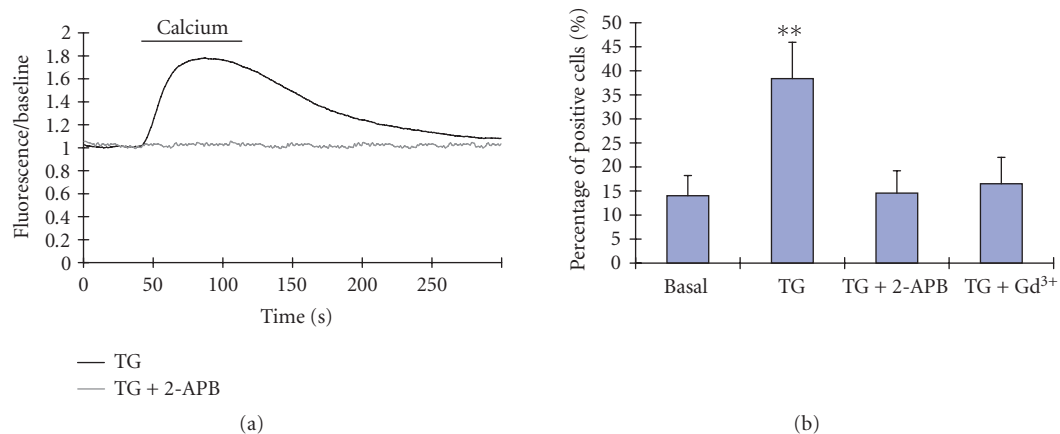


FIGURE 3: CRAC channel blockers prevented thapsigargin-evoked ROS production. (a) 2-APB suppressed the Ca²⁺ signal induced upon readmission of external Ca²⁺. The cells were pretreated for 8 minutes with thapsigargin (2 μM) in Ca²⁺-free solution, 20 μM 2-APB was added just 2 minutes before the readmission of the Ca²⁺ as indicated. Figure 3(a) is representative of three experiments performed on different experimental days. (b) 2-APB and Gd³⁺ inhibited the ability of thapsigargin (2 μM) to stimulate ROS production in the presence of external Ca²⁺. Three independent experiments have been performed. TG: thapsigargin. ***P* < .01 versus control group.

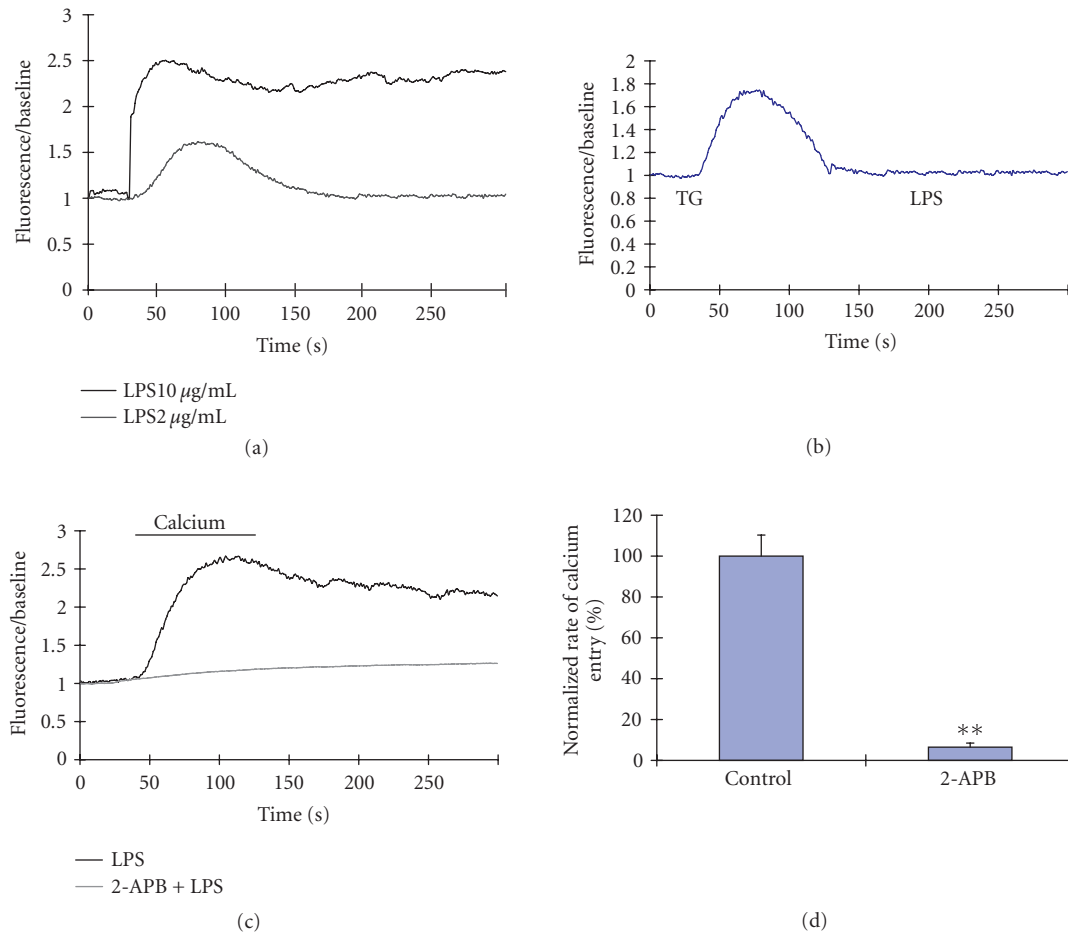


FIGURE 4: LPS stimulated Ca^{2+} entry and $[\text{Ca}^{2+}]_i$ increase through CRAC channels. (a) LPS induced an elevation in calcium in RAW264.7 cells in the presence of external calcium. (b) After 3 minutes of $1 \mu\text{M}$ thapsigargin treatment, the effect of LPS ($10 \mu\text{g/mL}$) on $[\text{Ca}^{2+}]_i$ increase was completely abolished. (c) $20 \mu\text{M}$ 2-APB suppressed the Ca^{2+} signal induced upon readmission of external Ca^{2+} to cells pretreated for 8 minutes with LPS ($2 \mu\text{g/mL}$) in Ca^{2+} -free solution. 2-APB was added just 2 minutes before the readmission of the Ca^{2+} . (d) The summarized aggregate data compares the effects of the control and 2-APB on the initial rate of Ca^{2+} entry following readmission of the Ca^{2+} to cells with stores depleted by LPS. The rate of Ca^{2+} influx was obtained by measuring the initial slope of the Ca^{2+} rise following readmission of Ca^{2+} to cells with depleted stores. 2-APB was added just 2 minutes before the readmission of the Ca^{2+} . Three independent experiments have been performed. Figures 4(a), 4(b), 4(c) are representative of at least three experiments performed on different experimental days. $**P < .01$ versus control group.

under the conditions imposed by the experiment, both LPS and thapsigargin induced an increase in intracellular $[\text{Ca}^{2+}]_i$, either in the presence or absence of Ca^{2+} in the extracellular medium. However, in the presence of external Ca^{2+} , the Ca^{2+} signal was sustained, and in the absence of external Ca^{2+} , thapsigargin only initiate a mild and less sustained rise in $[\text{Ca}^{2+}]_i$. Similar results were found in the LPS group. Our results indicated that the main source of increased intracellular Ca^{2+} was indeed extracellular. Secondly, the effect of LPS on $[\text{Ca}^{2+}]_i$ increase was completely abolished by pretreatment with thapsigargin indicating that LPS triggered the entry of extracellular Ca^{2+} via depleting sarco/endoplasmic reticulum Ca^{2+} stores. This subsequently activated CRAC channels just as did thapsigargin. Thirdly, the CRAC channel inhibitor, 2-APB, completely suppressed the Ca^{2+} entry signal evoked by thapsigargin, and suppressed approximately 93% of the Ca^{2+}

entry signal evoked by LPS. This shows that other Ca^{2+} channels also open when induced by LPS, but contribute little to the overall Ca^{2+} signal. 2-APB inhibited I_{CRAC} and store-operated entry in the mutant DT40 cell line in which InsP3 receptors are not expressed [14]. Hence, InsP3 receptors are not required for 2-APB block of store-operated entry. 2-APB is becoming a popular tool to probe functional consequences of inhibiting store-operated entry, because it seems to block CRAC channels directly and rapidly, most likely on an external site [14, 15]. The trivalent cation, Gd^{3+} , which fully blocks the channels in the low micromolar concentration range [16], is often used to separate endogenous CRAC channels from recombinant transient receptor potentials (TRP's) [17].

ROS are a molecular group that can be produced in the course of different physiological processes and react with a

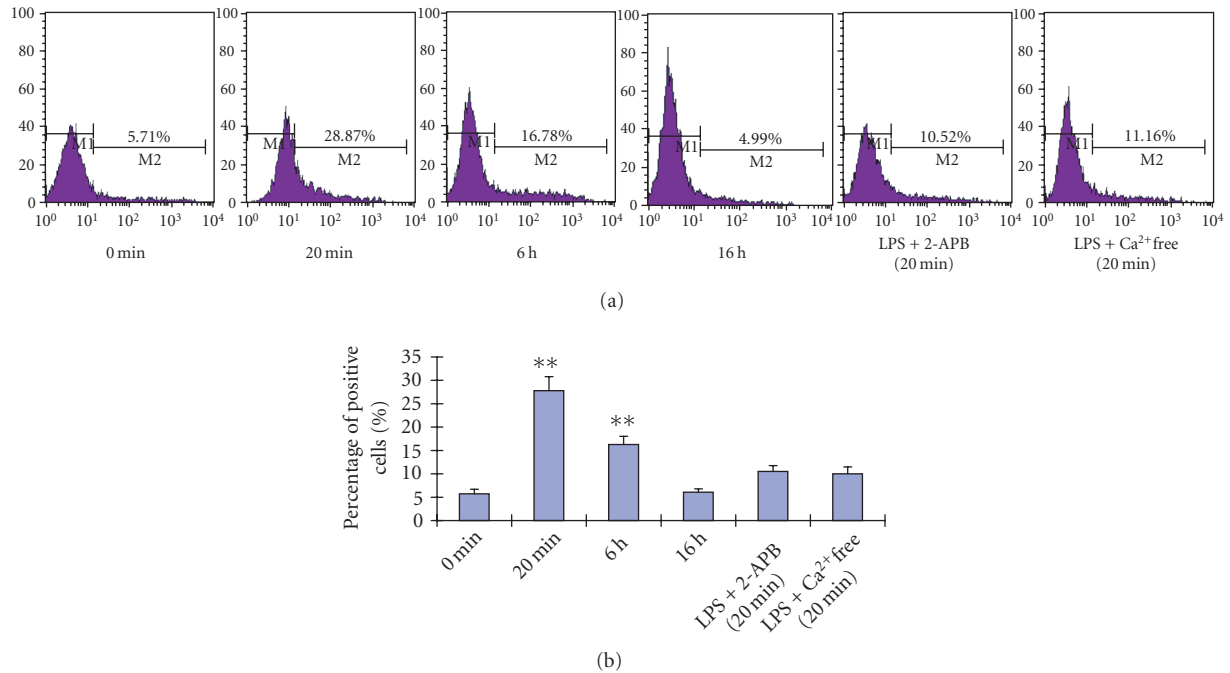


FIGURE 5: LPS-evoked Ca²⁺ influx stimulated ROS production. (a) Flow cytometry profiles showing the time course of ROS production following stimulation with LPS (10 µg/mL) in the presence of external Ca²⁺. Stimulation of ROS production with LPS (10 µg/mL) at 20 minutes was suppressed in the absence of external Ca²⁺ or when pretreated with 2-APB (20 µM) in the absence of external Ca²⁺. Figure 5(a) is representative of three experiments performed on different experimental days. (b) Aggregate data are summarized. TG: thapsigargin. ***P* < .01 versus control group.

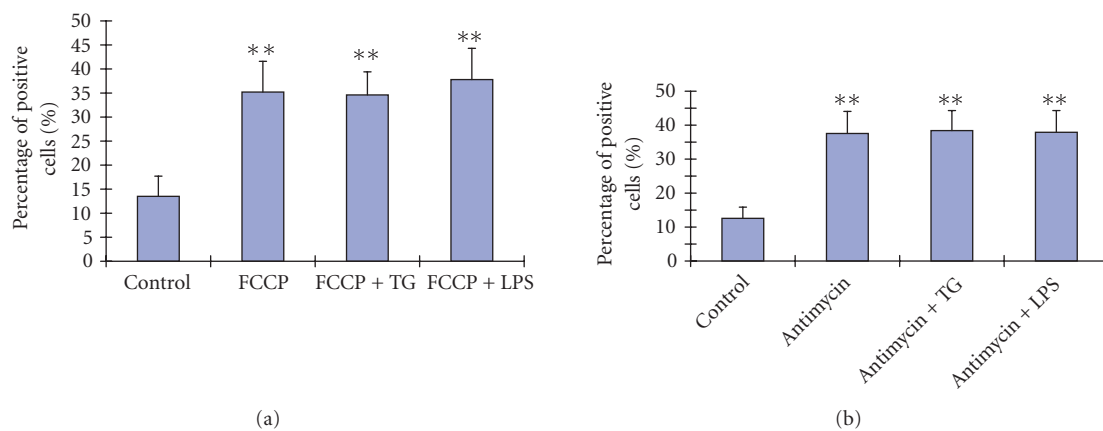


FIGURE 6: (a) Effect of mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) on thapsigargin and LPS-evoked generation of ROS. When the FCCP (1 mM) was added to the cells, a significant increase in ROS production was observed at 10 minutes. In the presence of FCCP, stimulation of cells with 2 µM TG or 10 µg/mL LPS failed to induce further increases in ROS level. (b) Effect of antimycin on thapsigargin and LPS-evoked generation of ROS. Treatment of cells with 5 mM antimycin led to a significant increase in ROS generation at 10 minutes. In the presence of the antimycin, stimulation of cells with 10 µg/mL LPS failed to evoke further increases in ROS production. A similar result was obtained when the cells were challenged with TG (2 µM) in the presence of the antimycin. Three independent experiments have been performed. TG: thapsigargin. ***P* < .01 versus control group.

large variety of oxidizable cellular components [18]. Therefore, reduction-oxidation reactions involving ROS have gained attention as important chemical processes with implications in cellular signal transduction, especially those involving macrophages. Under our experimental conditions,

in the presence of extracellular Ca²⁺, both thapsigargin and LPS induced an increase in ROS generation in a dose-dependent manner. However, the effects were completely abolished in the absence of extracellular Ca²⁺, demonstrating that ROS production is activated by Ca²⁺ influx, but

not by Ca^{2+} release from the stores, despite reasonable overall increases in cytosolic Ca^{2+} concentration. The results of many investigations have shown that calcium is essential for production of ROS. Elevation of intracellular calcium level is responsible for activation of ROS-generating enzymes and formation of free radicals by the mitochondria respiratory chain. Cytosolic Ca^{2+} is also an important regulator of NADPH oxidase activation leading to the generation of ROS, and ROS production is largely dependent on $[\text{Ca}^{2+}]_i$ mobilization. The mechanism of cytosolic Ca^{2+} activation NADPH oxidase involves PKC, phospholipase A_2 , and Rac pathways [19].

Next, we designed experiments to identify the nature of the Ca^{2+} entry pathway that drives ROS production. If Ca^{2+} entry through CRAC channels stimulates ROS release, then one would expect inhibitors of CRAC channels to suppress this release. We tested this by using the CRAC channel blockers 2-APB and Gd^{3+} . Our results showed that CRAC channel blockers completely suppressed the Ca^{2+} entry signal and also prevented the ability of ROS production evoked both by thapsigargin and LPS, demonstrating that Ca^{2+} entry through CRAC channels stimulates ROS release. Furthermore, the ROS release in macrophages did not require voltage-gated Ca^{2+} influx, as direct voltage-gated Ca^{2+} channel blockade with nifedipine, did not suppress the ROS production in our experiments. All these findings indicate that ROS production is the downstream consequence of CRAC channel activation and close functional coupling between CRAC and ROS production in murine macrophages. In our experiments, both thapsigargin and LPS induced an elevation in ROS at 1 minute. However, thapsigargin induced an elevation in ROS which reached maximal levels within 10 minutes and returned to basal levels in 20 minutes, while it reached maximal levels within 20 minutes and was still higher in 6 hours in the LPS group. The experiment shows that thapsigargin, a CRAC agonist, evoked a transient rise in ROS, indicating that Ca^{2+} entry through CRAC is a trigger for ROS production. Sustained production of ROS needs activation of signaling cascades following stimulation of LPS. In concordance with these results, thapsigargin triggered Ca^{2+} release from the internal stores, and the Ca^{2+} signal decayed back to prestimulation levels over several minutes (Figure 1(b)), however, the Ca^{2+} signal was sustained with 10 $\mu\text{g}/\text{mL}$ LPS (Figure 4(a)), these data clearly showed that the different time scale in ROS formation depends on different Ca^{2+} signal induced by thapsigargin and LPS.

Ca^{2+} -dependent release of ROS suggests a dominant role for the ROS generation enzyme that depended on Ca^{2+} in our experiments, so it was of interest to analyze the intracellular source of ROS. Mitochondria are probably the most important source of increased free radical production. These organelles accumulate large amounts of Ca^{2+} that can lead to the generation of ROS, being the basis of excitotoxicity injury mechanisms [20]. In the present study, we showed that the mitochondrial uncoupler, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone, and the inhibitor of the electron transport chain, antimycin, evoked a marked increase in ROS and completely inhibited thapsigargin and

LPS-evoked responses. These results are consistent with previous reports in mouse pancreatic acinar cells [21]. Taken together, these data suggest that ROS evoked by thapsigargin and LPS are generated mainly in the mitochondria.

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