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Role of Calcium in Tumor Necrosis Factor-α Production by Activated Macrophages[1]

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The role of calcium in the production of tumor necrosis factor-α (TNF-α) by the lipopolysaccharide (LPS)-stimulated macrophage cell line, J774.1, was investigated. Flow cytometric measurement of intracellular calcium concentration ($[Ca]_i$) using 2-(3,6-bis(acetyloxy)-2,7-dichloro-9*H*-xanthen-9-yl)benzoic acid (fluo-3)-loaded J774.1 cells revealed that LPS at more than 0.1 μg/ml increased $[Ca]_i$ transiently in the presence or absence of serum, and that a mixture of a calcium chelator, ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and a calcium release blocker from intracellular store sites, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), inhibited the $[Ca]_i$ response induced by LPS. In concordance with this, production of TNF-α was inhibited by EGTA and/or TMB-8. These reagents also reduced the level of TNF-α mRNA significantly. These results indicate that the transient increase of $[Ca]_i$ plays a role in LPS-induced expression of TNF-α by the macrophage cell line.

Key words: calcium, calcium chelator, lipopolysaccharide (LPS), macrophage, tumor necrosis factor-α (TNF-α).

Lipopolysaccharide (LPS) is well known to activate macrophages, causing the manifestation of a wide spectrum of biological functions, including the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (*1-3*). The molecular mechanisms underlying these effects, however, are not fully understood. Although a role of calcium has been suggested by the transient increase in the intracellular calcium concentration ($[Ca]_i$) in response to LPS (*4*) and by the effect of inhibitors of calmodulin-dependent protein kinase or protein kinase C (*5-7*), the heterogeneity in the $[Ca]_i$ response has made it difficult to define the role of calcium in LPS-induced activation of macrophages (*4*). Indeed there have been several reports describing no increase of $[Ca]_i$ using murine macrophages (*8-10*). Recently, a fluorescent probe, 2-(3,6-bis(acetyloxy)-2,7-dichloro-9*H*-xanthen-9-yl)benzoic acid, known as fluo-3, was introduced as a more sensitive tool for measuring $[Ca]_i$ with a flow cytometer (*11, 12*). Flow cytometric measurement, furthermore, has allowed definition of the heterogeneity of the $[Ca]_i$ response (*12*). Alternatively, the use of a macrophage cell line that can respond to LPS, such as the RAW 264.7 murine macrophage cell line (*13, 14*), may overcome the difficulty in studying $[Ca]_i$.

In this study we used the J774.1 cell line, because it also produces TNF-α in response to LPS (*15*). First we measured the $[Ca]_i$ response using fluo-3-loaded cells. We then examined the effects of a calcium chelator, ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and a calcium release blocker from intracellular store sites, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) (*13, 16, 17*), on LPS-induced TNF-α production.

MATERIALS AND METHODS

Cells—The J774.1 murine macrophage cell line was kindly provided by Dr. M. Nishijima (NIH, Tokyo), and maintained in RPMI-1640 containing 10% fetal calf serum (FCS; GIBCO/BRL, Gaithersberg, MD) (RPMI-1640 medium). Mouse fibroblast L·P3 cells were maintained in the same medium.

Cell Cultures—J774.1 cells, at a density of approximately 2.5×10^5 cell/well in a 24-well plastic plate, were rinsed with prewarmed RPMI-1640 medium, and then treated with 0.5 ml of RPMI-1640 medium containing EGTA, TMB-8 or a mixture of these two reagents at 37°C for 20 min. They were then stimulated with 10 μg/ml of LPS (*Escherichia coli*, 055:B5; Difco Laboratories, Detroit, MI) (1st culture). After harvesting the supernatant, the cells were rinsed with prewarmed RPMI-1640 medium twice, then restimulated with 10 μg/ml of LPS at 37°C for 6 h (2nd culture). Each supernatant was dialyzed against phosphate buffered saline (PBS) before assaying of TNF-α activity.

Assaying of TNF-α Activity—TNF-α activity was

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Abbreviations: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; $[Ca]_i$, intracellular calcium concentration; fluo-3, 2-(3,6-bis(acetoxy)-2,7-dichloro-9*H*-xanthen-9-yl)benzoic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TMB-8, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride; FCS, fetal calf serum; PBS, phosphate buffered saline; HBSS, Hanks' balanced salt solution; fluo-3-AM, fluo-3 acetoxymethyl ester; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RT-PCR, polymerase chain reaction of reverse-transcribed mRNA.

measured with the L·P3 cell line, a subline of L929 mouse fibroblasts, as target cells, according to the method previously described (*18*).

Measurement of $[Ca]_i$ *with a Flow Cytometer*—Changes in $[Ca]_i$ were measured using fluo-3-loaded J774.1 cells. For loading, the cells were washed with Hanks' balanced salt solution (HBSS) three times, resuspended at a cell density of 10^7 cells/ml in HBSS containing 4 mM fluo-3 acetoxymethyl ester (fluo-3-AM), then incubated at 37°C for 20 min. Then 5 volumes of HBSS containing 1% FCS was added, followed by incubation at 37°C for 20 min. The cells were then washed with HBSS three times, and adjusted to a cell density of 10^6 cells/ml in HBSS containing 10 mM HEPES (pH 7.2) and 1 mg/ml of bovine serum albumin. Before measurement, the cells were passed through a nylon mesh (mesh size: 50) to remove cell aggregates. Changes in $[Ca]_i$ were measured with FACScan (Becton Dickinson) using CHRONYS™ software (Becton Dickinson). The results were expressed as the mean channel of over 10,000 events, or as the maximal difference of the mean channels between experiment and control.

Metabolic Labeling of J774.1 Cells—J774.1 cells in 6-well plates were rinsed with 0.5 ml of Swim's S-77 (Sigma) supplemented with 0.42 mM calcium chloride and 2% FCS (Swim's S-77 medium), then treated with EGTA, TMB-8, or a mixture of the two reagents for 20 min. They were then stimulated with 10 μg/ml of LPS, and labeled by incubation at 37°C for 2 h with 50 μCi of L-[^{35}S]cysteine (specific activity, 1,100 Ci/mmol; 1 Ci=37 GBq, ICN Biochemicals, Costa Mesa, USA). The labeled cells were lysed with 0.5 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, containing 9 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM phenylmethanesulfonyl fluoride (PMSF), 50 μg/ml leupeptin, and 2 mM EGTA). The resultant cell lysates and culture supernatants, from which cell debris was removed by centrifugation, were immunoprecipitated (*19*) with rabbit anti-mouse TNF-α antiserum, which was kindly supplied by Drs. K. Matsushima and N. Mukaida (Cancer Research Institute, Kanazawa Univ., Kanazawa), and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (*20*). The gels were fixed and visualized by fluorography, using Enlightning (Du Pont-New England Nuclear). The radioactivity of each band in the gels was quantitated with a Bioimage analyzer (Fuji Photo Film).

Semi-Quantitative Polymerase Chain Reaction of Reverse-Transcribed mRNA (RT-PCR)—J774.1 cells cultured in 10-cm dishes were treated with EGTA, TMB-8, or a mixture of the two reagents for 20 min, then stimulated with 10 μg/ml LPS for 2 h as described above.

Total RNA was prepared using a nucleic acid extraction kit (Sepa-Gene; Sanko Junyaku). Five micrograms of RNA was mixed with 120 ng of oligo d(T) primer, heated at 65°C for 5 min, cooled to room temperature slowly, then reverse-transcribed at 37°C for 1 h in a final volume of 20 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM of each dNTP, 16 units of ribonuclease inhibitor (TOYOBO), and 8 units of Molony murine leukemia virus reverse transcriptase (TOYOBO). One microliter of cDNA thus prepared was mixed with 18 μl of the reaction buffer containing 0.4 μmol of Tris-HCl (pH 8.3), 1 μmol of KCl, $MgCl_2$ at the optimal concentration, 4 μmol of each dNTP, and 4.5 pmol of both sense-strand and antisense-strand primers. The mixture was heated at 94°C for 5 min and thereafter at the optimal annealing temperature for 5 min, followed by the addition of 0.5 unit (1 μl) of Taq DNA polymerase (Gibco/BRL). Amplification was carried out at 72°C for 2 min, 94°C for 30 s, and the optimal annealing temperature for 1 min for 30 cycles. The PCR products were then analyzed by PAGE.

The mouse TNF-α-specific sequence was amplified using a sense-strand primer (residues 280-299) and an antisense-strand primer (residues 420-401), which yielded a 141-bp product (*21*). The annealing temperature was 60°C, and the optimal concentration of $MgCl_2$ was 2 mM. The mouse β_2-microglobulin (β_2m)-specific sequence was amplified using a sense-strand primer (residues 47-66) and an antisense-strand primer (residues 3160-3141), which yielded a 220-bp product (*22*). The annealing temperature was 60°C, and the optimal concentration of $MgCl_2$ was 1 mM.

Semi-quantitation of mRNA was performed according to the method described previously (*23*). Serially diluted cDNAs were amplified for the β_2m-specific sequence. The intensity of each PCR product in the polyacrylamide gels after staining with ethidium bromide was measured using NIH Image 1.55. The differences in intensity among the gels were normalized relative to the intensities of the bands of molecular weight standards. The results were expressed as % of β_2m.

RESULTS

Although TNF-α production in response to LPS had been reported in J774.1 cell line (*15*), we first examined whether our J774.1 cell line produced TNF-α in response to LPS. As shown in Fig. 1, the cells responded to LPS in a dose-dependent manner by producing TNF-α activity. We therefore used this cell line for studying the role of calcium in LPS-induced TNF-α production. Since it is well known that serum enhances LPS response, we then examined the effect of serum on LPS-induced TNF-α production. As also shown in Fig. 1, in the presence of serum, 0.01 μg/ml of LPS was required for production of TNF-α, whereas, in the

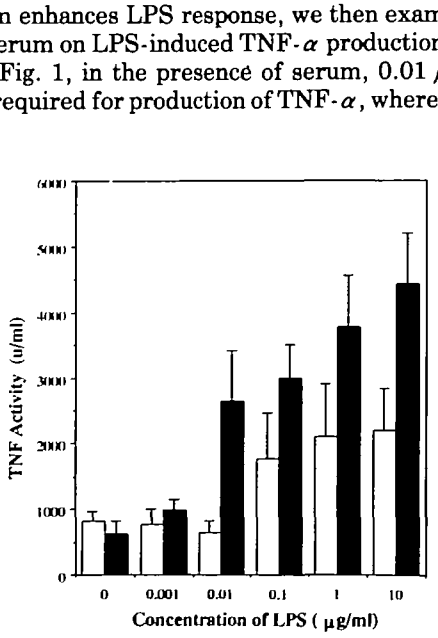

Fig. 1. **Production of TNF-α on LPS stimulation.** J774.1 cells were stimulated with various concentrations of LPS in the presence (black bars) or absence (white bars) of 10% serum for 2 h, then TNF-α activity in each supernatant was assayed.

absence of serum, 0.1 μg/ml of LPS was required.

We then measured $[Ca]_i$ with a flow cytometer using fluo-3-loaded J774.1 cells. Figure 2 illustrates the $[Ca]_i$ response of the cells. LPS induced a transient increase in $[Ca]_i$, whereas A23187 induced a sustained increase in $[Ca]_i$. As shown in Fig. 3, the $[Ca]_i$ response was dependent on the concentration of LPS, and in the absence of serum 0.1 μg/ml of LPS was minimally required to induce a detectable $[Ca]_i$ response. This was confirmed by three independent measurements, and, even in the presence of serum, 0.1 μg/ml of LPS was also minimally required to induce the $[Ca]_i$ response (data not shown). Since LPS at 10 μg/ml gave a good $[Ca]_i$ response (Fig. 3), we tested the effects of a calcium chelator (EGTA) and a calcium release blocker from intracellular store sites (TMB-8) on LPS-induced $[Ca]_i$ response at this concentration. As shown in Fig. 2, a mixture of these inhibitors blocked the $[Ca]_i$ increase by 96%. On the other hand, TMB-8 alone blocked the $[Ca]_i$ increase by 47%, whereas EGTA alone marginally blocked the $[Ca]_i$ increase.

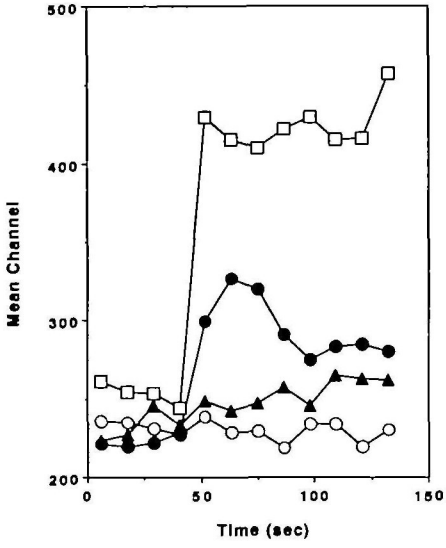

Fig. 2. **Measurement of $[Ca]_i$ with a flow cytometer.** J774.1 cells were loaded with fluo-3-AM, then incubated in the absence or the presence of a mixture of EGTA (2 mM) and TMB-8 (50 μM) for 2 min. The $[Ca]_i$ response by the cells was then measured, followed by stimulation with LPS (10 μg/ml) or A23187 (5 μg/ml) after 40.5 s (3 intervals). The data for cells treated with A23187, LPS, and a mixture of EGTA and TMB-8 are denoted by open squares, closed circles, and closed triangles, respectively. The data for unstimulated cells are denoted by open circles.

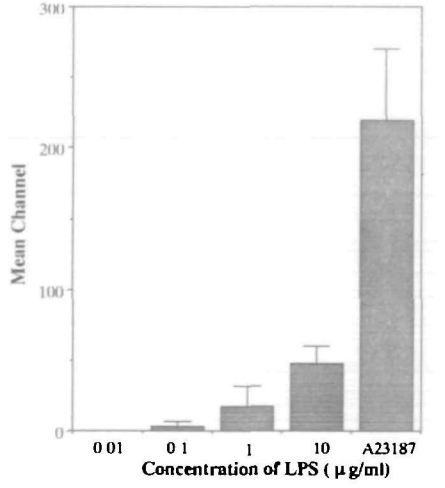

Fig. 3. **$[Ca]_i$ response with various concentrations of LPS.** The $[Ca]_i$ response by J774.1 cells preloaded with fluo-3-AM was measured with a flow cytometer, then cells were stimulated with various concentrations of LPS. In this case, the cells were suspended in RPMI-1640 medium without serum. Determination was carried out in triplicate. The results were expressed as the mean ± standard error of the difference between peak channel (experiment) and base line (control).

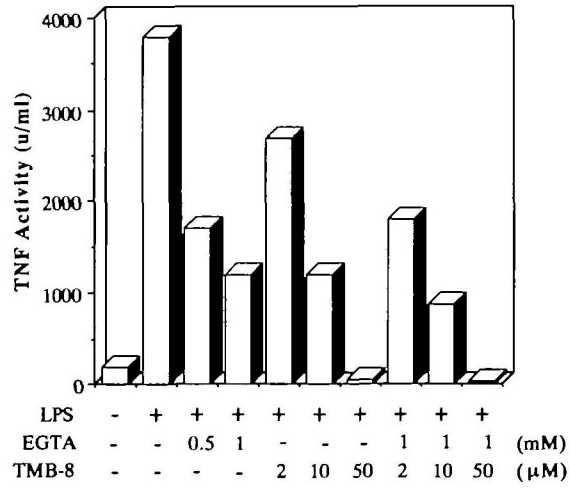

Fig. 4. **Inhibition of TNF-α production by EGTA, TMB-8, and a mixture of them in LPS-stimulated cells.** J.774.1 cells were treated with EGTA, TMB-8, or a mixture of the two reagents, then stimulated with LPS (10 μg/ml) for 2 h. Each supernatant was dialyzed against phosphate buffered saline (PBS), then its TNF-α activity was assayed.

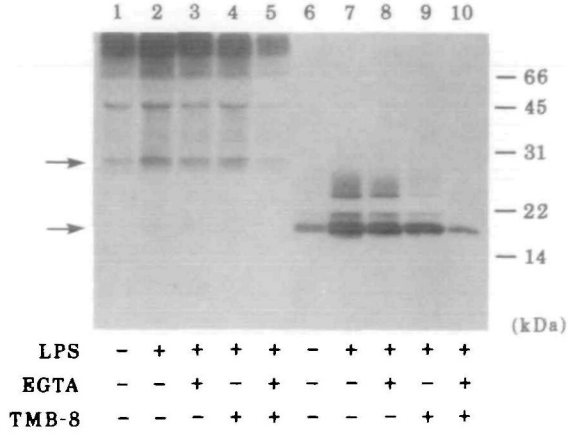

Fig. 5. **Analysis of metabolically labeled TNF-α.** J774.1 cells were treated with 1 mM EGTA, 50 μM TMB-8, or a mixture of the two reagents, cultured in the absence (−) or presence (+) of LPS (10 μg/ml), then labeled for 2 h with L-[^{35}S]cysteine. The resultant cell lysates (lanes 1 to 5) and culture supernatants (lanes 6 to 10) were immunoprecipitated with anti-TNF-α antiserum and then analyzed by SDS-PAGE.

TABLE I. **Quantitation of radioactivity of pre-TNF-α and mature TNF-α.**

Inhibitor[a]	LPS	Cell lysate[b]		Supernatant[b]	Total[c]	% processing[d]	% release[e]
		26 kDa	17 kDa	17 kDa			
−	−	100	23	287	350 (19)	89	82
−	+	212	49	1755	1889 (100)	96	93
EGTA	+	175	39	1043	1152 (61)	94	91
TMB-8	+	170	38	590	696 (37)	90	85
EGTA+TMB-8	+	91	15	149	200 (11)	82	74

[a]EGTA and TMB-8 were used at the concentrations of 1 mM and 50 μM, respectively. [b]The radioactivity of each band in the gel shown in Fig. 3 was quantitated with a Bioimage analyzer and expressed in arbitrary units. [c]Total radioactivities of 26- and 17-kDa TNF-α in both cell lysates and supernatants are shown after the radioactivity of 26-kDa TNF-α was corrected for the difference in the number of cysteine residues (5 residues for 26-kDa TNF-α; 2 residues for 17-kDa TNF-α). The ratios of total radioactivities to those in LPS-stimulated cells without inhibitors are given in parentheses. [d]The extent of processing was expressed as % processing according to the following formula: (Radioactivity of 17-kDa TNF-α in both cell lysates and supernatants)/(total radioactivity of 26- and 17-kDa TNF-α)×100. [e]The extent of release was expressed as % release according to the following formula: (Radioactivity of 17-kDa TNF-α in supernatants)/(total radioactivity of 26- and 17-kDa TNF-α)×100.

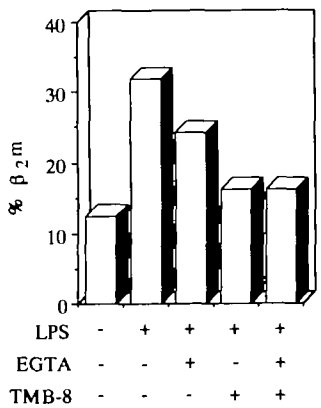

Fig. 6. **Expression of TNF-α mRNA.** J774.1 cells were treated with 1 mM EGTA, 50 μM TMB-8, or a mixture of the two reagents, cultured in the absence (−) or presence (+) of LPS (10 μg/ml) for 2 h, then subjected to RT-PCR. The intensities of the bands of PCR products in the polyacrylamide gel after staining with ethidium bromide were measured with the aid of NIH Image software. The data are expressed as % of β_2m.

To examine the role of calcium in TNF-α production, J774.1 cells were stimulated with LPS for 2 h in the presence of EGTA, TMB-8, and a mixture of them. As shown in Fig. 4, both these reagents significantly inhibited TNF-α production. Here we used LPS at 10 μg/ml, because we aimed at examining the production of TNF-α under the exactly same conditions as those giving the good $[Ca]_i$ response.

To determine whether the inhibition of TNF-α production by these reagents is due to a block in the process of protein synthesis or proteolytic processing, cell-associated and extracellular TNF-α labeled with L-[^{35}S]cysteine were immunoprecipitated and analyzed by SDS-PAGE. After quantitation of the radioactivities in pre-TNF-α (26 kDa) and mature TNF-α (17 kDa), and correction for the difference in the number of cysteine residues (5 residues for 26-kDa TNF-α; 2 residues for 17-kDa TNF-α), the percentage of processing or release was calculated with the following formulae: (radioactivity of 17-kDa TNF-α in both cell lysates and supernatants)/(total radioactivity of 26- and 17-kDa TNF-α)×100 (for processing); and (radioactivity of 17-kDa TNF-α in supernatants)/(total radioactivity of 26- and 17-kDa TNF-α)×100 (for release). As shown in Fig. 5 and Table I, the total amounts of 26- and 17-kDa TNF-α decreased with EGTA, TMB-8 or a mixture of them, this being compatible with the results of the inhibition of TNF-α production by these inhibitors. It should be noted that neither the processing nor the release was influenced by these inhibitors (Table I).

The expression of TNF-α mRNA was also examined by RT-PCR. As shown in Fig. 6, the expression of TNF-α mRNA induced by LPS was inhibited by treatment with EGTA and/or TMB-8, indicating that calcium-dependent pathways are involved in the induction of TNF-α mRNA by LPS.

The above findings raised the possibility that more $[Ca]_i$ might enhance the production of TNF-α. We therefore examined whether the addition of A23187 enhanced the production of TNF-α by LPS. Contrary to our expectation, A23187 reduced the production of TNF-α by LPS from 16,000 to 6,000 u/ml after culture for 20 h.

DISCUSSION

Using the J774.1 murine macrophage cell line, we demonstrated that LPS induced the expression of TNF-α mRNA and a transient $[Ca]_i$ response, both of which could be inhibited by a mixture of EGTA and TMB-8. Since the addition of A23187 reduced the production of TNF-α by LPS, an adequate level and/or a transient increase in $[Ca]_i$ may be required for TNF-α production.

Although a role of calcium has been suggested by the transient increase of $[Ca]_i$ in response to LPS (*4*) and by the effect of an inhibitor of calmodulin-dependent protein kinase or protein kinase C (*5-7*), to our knowledge the effect of inhibitors on both TNF-α production and the $[Ca]_i$ response were examined under the same conditions only in the present work and one other study (*24*).

There are several reports describing the absence of an increase in $[Ca]_i$ using murine macrophages (*8-10*). In our J774.1 murine macrophage cell line, serum enhanced the LPS-induced production of TNF-α but not the LPS-induced $[Ca]_i$ response. It is conceivable, therefore, that LPS-CD14 interaction may not cause the $[Ca]_i$ response, which would be compatible with the report by Martin *et al.* (*10*). Since there are reports that even 10 μg/ml of LPS provoked no $[Ca]_i$ response in normal mouse macrophages (*8, 9*), a high concentration of LPS does not always lead to the $[Ca]_i$ response. It should be pointed out here that

normal mouse macrophages were heterogenous with regard to the $[Ca]_i$ response upon exposure of 1 μg/ml of LPS, with approximately half of the macrophages showing the response (*4*). Therefore, it is suggested that the use of a homogeneous cell line such as J774.1 cells may be critical for detection of the $[Ca]_i$ response, although in normal macrophages a special loading condition was required for successful detection of the $[Ca]_i$ response (*4*). Aequorin-loaded RAW 264.7 cells have been reported to show no $[Ca]_i$ response with 0.05 μg/ml of LPS (*13*), presumably because the concentration of LPS was not high enough to induce a detectable $[Ca]_i$ response.

Although calcium is believed to play a critical role in signal transduction, it may also play important roles in proteolytic processing and secretion (*25*). The present study strongly indicated that EGTA and/or TMB-8 inhibited TNF-α production in response to LPS mainly at transcription, but not at proteolytic processing or secretion, although our results do not formally exclude the possibility that calcium is involved in proteolytic processing and secretion in LPS response.

Although the present study suggested that calcium plays a role in the induction of TNF-α mRNA by LPS, it was important to exclude the possibility that the inhibitory effects of EGTA and TMB on TNF-α production were merely due to cellular toxicity. Since the treatment of J774.1 cells with LPS for 2 h did not induce unresponsiveness to LPS (data not shown), cells were further treated with LPS for 6 h after stimulation for 2 h in the presence of the inhibitor(s), and then TNF-α activity was measured. These cells produced almost the same amount of TNF-α activity irrespective of the inhibitor treatment in the first culture (data not shown), negating the possibility that EGTA and/or TMB-8 merely affected cell viability.

A number of studies have focused on the calcium-dependent intracellular processes that mediate the LPS response, such as the activation of protein kinases (*6, 7, 15, 26, 27*) and the phosphorylation of intracellular proteins. However, the role of a $[Ca]_i$ increase in cytokine production induced by LPS has not been fully examined. The present study showed unequivocally that the LPS-mediated transient increase in $[Ca]_i$ plays a critical role in the induction of TNF-α mRNA expression. Since PAF antagonists were reported to inhibit the $[Ca]_i$ increase in guinea pig macrophages without affecting TNF-α production (*24*), it will be interesting to examine whether J774.1 cells also utilize the PAF receptor for LPS signaling. Although much work is required to determine how the intracellular $[Ca]_i$ level is increased by LPS, the inhibitory effects of EGTA and TMB-8 suggest that the release of calcium from intracellular store sites and/or the entry of extracellular calcium may be involved in the increase.

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