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Role of intracellular calcium as a priming signal for the induction of nitric oxide synthesis in murine peritoneal macrophages

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SUMMARY

Because the role of intracellular Ca^{2+} in the two-signal process for the induction of nitric oxide (NO) synthesis is controversial, this study was undertaken to examine the role of Ca^{2+} in the transcriptional regulation of inducible NO synthase (iNOS) in murine peritoneal macrophages. Treatment of the cells with thapsigargin (TG) or 2,5-di-(t-butyl)-1,4-benzodihydroquinone (tBuBHQ), which are the specific and potent Ca^{2+}-ATPase inhibitors of endoplasmic reticulum (ER), showed modest effects on tumoricidal function, whereas TG or tBuBHQ in combination with interferon-γ (IFN-γ) or lipopolysaccharide (LPS) showed marked effects on tumoricidal function of the cells. The tumoricidal effects of the activated macrophages were correlated with the amount of NO synthesis, and totally abrogated by the use of NOS inhibitor, N^G-monomethyl-L-arginine (N^GMMA). The increases in NO synthesis was reflected as increased amounts of iNOS mRNA by Northern blotting. To confirm that iNOS induction was due to the changes in the intracellular Ca^{2+} level, the acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA-AM), an intracellular Ca^{2+} chelator, was used. Blocking the increase of cytosolic free Ca^{2+} significantly decreased the induction of NO synthesis. To demonstrate that intracellular Ca^{2+} acts as a 'priming' signal rather than a 'triggering' signal on the induction of NO synthesis by murine peritoneal macrophages, we designed several experiments. When the cells were treated with TG 6 hr after the treatment with IFN-γ, there was no increase in NO synthesis. In addition, when the cells were treated with TG or LPS 6 hr after treatment with tBuBHQ, a synergistic increase on NO synthesis was shown only in the case of LPS. When phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, was added to the cells 6 hr after the treatment with TG, there was a marked co-operative induction of NO synthesis, even though PMA alone has no effect. Based on the results obtained in this study, we suggest that cytosolic Ca^{2+} might be enough for the expression of iNOS gene as a priming signal and PKC might be involved in the induction of NO synthesis as a triggering signal by post-transcriptional modification of iNOS mRNA or iNOS itself in the activated murine peritoneal macrophages.

INTRODUCTION

Recent studies have demonstrated the crucial role of nitric oxide (NO) in the antimicrobial and tumoricidal activities of murine macrophages.[1,2] NO is released from activated macrophages by the oxidation of the terminal guanidino nitrogen atom of L-arginine by an inducible enzyme, NO synthase (NOS). NOS mRNA expression and enzyme activity in macrophages are induced by cytokines such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and interleukin-2 (IL-2).[3–5] However, macrophage activation capable of maximal NO production is induced by these cytokines only in the presence of bacterial lipopolysaccharide (LPS), which presumably provide a cosignal.[6,7]

Macrophage activation for induction of NO synthesis is a two-signal process. It is initiated by the binding of IFN-γ to its specific receptor[8] which results in a number of biochemical and functional alterations of the macrophages rendering them sensitive to triggering agent such as LPS.[9,10] Although, macrophage NOS can bind calmodulin tightly without a requirement for elevated intracellular Ca^{2+},[11] the major mechanism of induction of NOS in murine macrophages is

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Abbreviations: BAPTA-AM, acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; ER, endoplasmic reticulum; iNOS, inducible NOS; NO, nitric oxide; NOS, NO synthase; PKC, protein kinase C; tBuBHQ, 2,5-di-(t-butyl)-1,4-benzohydroquinone; TG, thapsigargin.

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known to be transcriptionally regulated.[12] In most cells, various physiological responses can result from synergistic interactions between Ca^{2+} signal and protein kinase pathways. Recent reports demonstrate that protein kinase C (PKC) might not be directly involved in the expression of NO synthase, instead they might be involved in the stabilization of the inducible NOS (iNOS) mRNA already transcribed by the treatment of IFN-γ.[13,14]

It has been shown that IFN-γ or A23187 as a priming signal causes changes in intracellular Ca^{2+} concentration, and that LPS, a most efficient NOS triggering agent, does not give rise to detectable calcium movements.[15–17] Therefore, precise molecular mechanisms involved in transduction of the activation signal for NO synthesis remain to be defined. Because the understanding of the role of cytosolic Ca^{2+} in the signal transduction pathway might be crucial, we tested whether intracellular free Ca^{2+} could act as a priming signal in the induction of NO synthesis from murine peritoneal macrophages. We used thapsigargin (TG) and 2,4-di-(t-butyl)-1,4-benzohydroquinone (tBuBHQ) as intracellular Ca^{2+} modulators and phorbol 12-myristate 13-acetate (PMA), a biologically active phorbol ester as a PKC activator in addition to IFN-γ and LPS. This study provides evidence that elevation of intracellular Ca^{2+} concentration acts as a 'priming' signal for the expression of iNOS mRNA and PKC activation acts as a 'triggering' signal for the synergistic induction of NO synthesis by post-transcriptional regulation of iNOS.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were used between 8 and 12 weeks of age. The mice were maintained in the Department of Microbiology and Immunology, Wonkwang University School of Medicine (Iksan, Korea).

Reagents

Murine recombinant rIFN-γ (1×10^6 U/mg) was purchased from Genzyme (Munich, Germany). Thioglycollate broth (Brewer) was purchased from Difco (Detroit, MI). LPS (phenol extracted *Salmonella enteritidis*), sodium nitrite, sulphanilamide, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, L-arginine, flavin-adenine dinucleotide, dithiothreitol (DTT), the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), lactate dehydrogenase, LiCl, urea, TG, and tBuBHQ were purchased from Sigma Chemical Co. (St. Louis, MO). DuPont New England Nuclear (Boston, MA) was the source of [α-^{32}P]dCTP. N^G-monomethyl-L-arginine (N^GMMA), tetrahydrobiopterin (BH_4) and the acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM) were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Bacterial nitrate reductase was kindly provided by Dr John B. Hibbs, Jr. (Department of Medicine, University of Utah, College of Medicine, and Veterans Administration Medical Center, Salt Lake City, UT). Ninety-six-well tissue culture plates and 100-mm diameter plastic petri dishes were purchased from Nunc Inc. (North Aurora Road, IL). Dulbecco's modified Eagle's minimum essential medium (DMEM) containing L-arginine (84 mg/l), fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). All reagents and media for tissue culture experiments were tested for their LPS content using a colorimetric *Limulus* amoebocyte lysate assay (detection limit, 10 pg/ml; Whittaker Bioproducts, Walkersville, MD).

Macrophage culture

Thioglycollate-elicited macrophages were harvested 3 days after intraperitoneal injection of 2·5 ml thioglycollate into mice and isolated as reported previously.[3] Peritoneal lavage was performed by using 8 ml of HBSS containing 10 U/ml heparin. When erythrocytes were visible, the cell pellet was treated with 0·2% NaCl for 30 seconds. Cells were then distributed in DMEM, which was supplemented with 10% (v/v) FCS, in either 96-well tissue culture plates (2×10^5 cells/well) or 100-mm diameter plastic Petri dishes (1×10^7 cells/dish), incubated for 3 hr at 37° in an atmosphere of 5% CO_2. Non-adherent cells were removed by suction, and then freshly prepared complete media were added with the indicated experimental reagents.

Macrophage cytotoxicity assay

Killing of ^{51}Cr-labelled K562 leukemia cells was measured using an 18-hr ^{51}Cr release assay.[18] K562 target cells were labelled for 1 hr at 37° with 500 μCi of ^{51}Cr/5×10^6 cells (sodium chromate; ICN Biomedicals, Inc., Irvine, CA), washed by centrifugation, allowed to 'leak' for 1 hr at 37° in complete DMEM containing 10% FCS, and washed again just before addition to macrophage cultures. Non-adherent cells were removed by washing three times after plating and the adherent macrophages were cultured for 24 hr with a medium alone or with medium containing the indicated stimuli. Quadruplicate wells were used for each culture condition. The media were then removed, the cells were washed twice, and 450 μl of fresh medium were added to each well. The ^{51}Cr-labelled K562 cells (2×10^5) were then added to each well in a volume of 50 μl. After 18 hr at 37° 100 μl of supernatants were removed and assayed for radioactivity in a gamma-spectrophotometer. Results are expressed as percentage specific ^{51}Cr release (percentage cytotoxicity) as calculated by the following formula: % specific cytotoxicity = 100 × (experimental c.p.m. − spontaneous c.p.m.)/(total c.p.m. − spontaneous c.p.m.). Total c.p.m. were obtained from lysis of 50 μl ^{51}Cr-labelled K562 cells (2×10^5) with 200 μl of 0·5% sodium dodecyl sulphate. Spontaneous release was determined from ^{51}Cr-labelled K562 cells incubated with medium alone. Spontaneous release was typically 30–35% of total c.p.m.

Preparation of cell lysates

Macrophage monolayers were washed three times with phosphate-buffered saline (PBS), scraped into PBS, and centrifuged at 500 *g* for 15 min at 4°. The cell pellet was resuspended in 500 μl of sonication buffer, which contained 40 mM Tris buffer (pH 8), 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 μg/ml aprotinin, and 100 μM phenyl methyl sulphonyl fluoride (PMSF), and lysed by sonication.[19] Aliquots of the lysate were used for Bradford protein assay (Bio-Rad, Richmond, CA) and iNOS enzyme activity was assayed.

Measurements of iNOS enzymatic activity

iNOS enzyme activity was measured as described.[20] Briefly, 50 μg of macrophage lysate were incubated for 2 hr at 37° in

20 mM Tris–HCl (pH 7·9) containing 4 μM BH_4, 4 μM flavin-adenine dinucleotide, 3 mM DTT, and 2 mM each L-arginine and the reduced form of NADPH. The reaction was initiated by adding L-arginine and NADPH and stopped by adding lactate dehydrogenase to oxidize residual NADPH. Product nitrite was measured by the Griess reaction.

Measurement of NO_2^- and NO_3^- concentration

Experiments were undertaken on cells grown either under standard conditions or in the presence of TG or tBuBHQ with or without LPS for 48 hr under the conditions described in the legend to Fig. 4. Supernatants in cultured macrophages were collected and mixed with an equal volume of the Griess reagent (1% sulphanilamide/0·1% *N*-(1-naphthyl)-ethylene-diamine dihydrochloride/2·5% H_3PO_4) and incubated for 10 min at room temperature.[21] Concentration of NO_2 was determined by measuring the absorbance at 540 nm in a Titertek Multiskan (Flow Laboratories, Australia) ELISA reader. The $NaNO_2$ was used as a standard. Cell-free medium alone contained 5–8 μM of NO_2^-; this value was determined in each experiment and subtracted from the value obtained with cells.

Preparation of probe

To detect iNOS mRNA transcripts, sense and antisense oligonucleotide primers specific for the coding regions of that gene were synthesized as described previously[14] DNA probes were radiolabelled by random priming with [α-^{32}P]dCTP according to standard protocols.[22] The resultant specific activity was approximately 1×10^8 c.p.m./μg and was used at 1×10^7 c.p.m./blot.

Northern blot analysis

Total RNA was isolated by using the LiCl–urea method,[23] subjected to electrophoresis in 1·2% agarose–formaldehyde gels, and transferred to nylon membranes by capillary action in 20 × SSC (1 × SSC = 0·15 M NaCl and 0·015 M sodium citrate, pH 7·2). After prehybridization, the filters were hybridized with random [α-^{32}P]dCTP-labelled probes having specific activity of $1–5 \times 10^8$ c.p.m./μg in 10% dextran sulphate, 50% formamide, 4 × SSC, 1 × Denhardt's solution, and 10 μg/ml salmon sperm DNA for 24 hr at 42°. Then the filters were washed, dried, and examined by autoradiography.[24]

RESULTS

Effects of TG on the tumoricidal activity of murine peritoneal macrophages.

Initially, we wished to determine whether endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitors could activate macrophages for tumoricidal activity. It is known that TG inhibit specifically the ER Ca^{2+}-ATPase and causes a discharge of the intracellular Ca^{2+} pool.[25,26] Thiglycollate-elicited macrophages for C57BL/6 mice were cultured for 24 hr with LPS (10 ng/ml), TG (300 nM) in the presence or absence of rIFN-γ (5 U/ml). As described in the Materials and Methods, the cells were washed twice and cultured for an additional 18 hr with fresh media containing ^{51}Cr-labelled K562 leukemia cells. Release of ^{51}Cr into culture supernatants was used to calculate the percentage of K562 cell lysis. As shown in Table 1, TG alone weakly induced tumoricidal activity, whereas TG in combination with rIFN-γ synergized to induce high and comparable tumoricidal activity. These data suggest that TG can prime murine peritoneal macrophages for tumoricidal activity. We also tested whether N^GMMA (2 mM) could block tumoricidal activity. N^GMMA completely abrogated macrophage-mediated cytolysis, indicating that NO is an essential mediator of TG plus rIFN-γ-activated macrophages (Table 1).

Table 1. Synergistic co-operation between TG and rIFN-γ to induce tumoricidal activity in murine peritoneal macrophages*

Treatment	Cytotoxicity (%) rIFN-γ (−)	rIFN-γ (+)
None	0	2 ± 3·1
+ N^GMMA†	ND‡	0
LPS	14 ± 3·1	88 ± 5·7
+ N^GMMA	4 ± 3·8	7 ± 3·4
TG	15 ± 3·4	81 ± 7·3
+ N^GMMA	3 ± 2·9	8 ± 2·6

* Thioglycollate-elicited macrophages were cultured with a medium alone, 10 ng/ml LPS, 300 nM TG in the presence (+) or absence (−) of 5 U/ml rIFN-γ. After 24 hr the macrophages were washed twice and cultured for an additional 18 hr with fresh media containing ^{51}Cr-labelled K562 leukemia cells. Release of ^{51}Cr into culture supernatants was used to calculate the percentage of K562 cell lysis. Values are means ± SD of three experiments.

† N^GMMA = 2 mM.

‡ ND = not determined.

Effects of TG or tBuBHQ on the production of NO by macrophages

Then, we examined whether TG could produce NO, either alone or in combination with IFN-γ or LPS from murine peritoneal macrophages. Thioglycollate-elicited macrophages were cultured either in medium alone or in medium that contained TG or tBuBHQ (5 μM). Then, cells were stimulated with rIFN-γ or LPS during a 48-hr culture, and then NO release was measured by using the method of Griess (nitrite and nitrate). As shown in Table 2, cells treated with TG or tBuBHQ alone modestly produced NO, and non-treated cells produced negligible amounts of NO (< 5 μM). Stimulation of the cells in combination with IFN-γ or LPS resulted in the increased accumulation of nitrite in the medium. These results further indicate that the elevation of cytoplasmic free Ca^{2+} by TG or tBuBHQ can prime murine peritoneal macrophages for NO synthesis. To discover the role of Ca^{2+} on the regulation of iNOS gene expression, iNOS mRNA was measured after the treatment of the cells with TG. Total cytoplasmic RNA was extracted from cultured cells as described in the legend to Fig. 1, separated by denaturing agarose gel electrophoresis, and determined by Northern hybridization with radiolabelled cDNA that encoded iNOS. In unstimulated macrophages, iNOS mRNA was not detectable (Fig. 1, a and b). TG alone

Table 2. Effect of TG and tBuBHQ on the induction of NO synthesis in murine peritoneal macrophages

Treatment*	NO_2^- (NO_2^- plus NO_3^-) secretion (μM)†		
	None	+ IFN-γ‡	+ LPS‡
None	< 5	16 ± 3 (37 ± 7)	14 ± 3 (30 ± 5)
TG (300 nM)	16 ± 2 (31 ± 6)	68 ± 5 (134 ± 11)	75 ± 6 (115 ± 14)
tBuBHQ (5 μM)	15 ± 3 (32 ± 7)	61 ± 5 (126 ± 10)	69 ± 7 (149 ± 11)

* Thioglycollate-elicited macrophages were cultured for 48 hr either in a medium alone or in a medium that contained TG or tBuBHQ.

† The amount of NO_2^- or NO_2^- plus NO_3^- released by macrophages was measured after 48 hr of incubation. Values are the means ± SD of four experiments.

‡ The cells were stimulated with 5 U/ml rIFN-γ or 10 ng/ml LPS.

had a small effect; however, when it was combined with either IFN-γ or LPS, iNOS mRNA content was increased greatly in comparison with the mRNA contents of TG- or rIFN-γ-treated cells. These results suggest that the increment of Ca^{2+} in cytosol is crucial as a 'priming' signal for the expression of the iNOS gene.

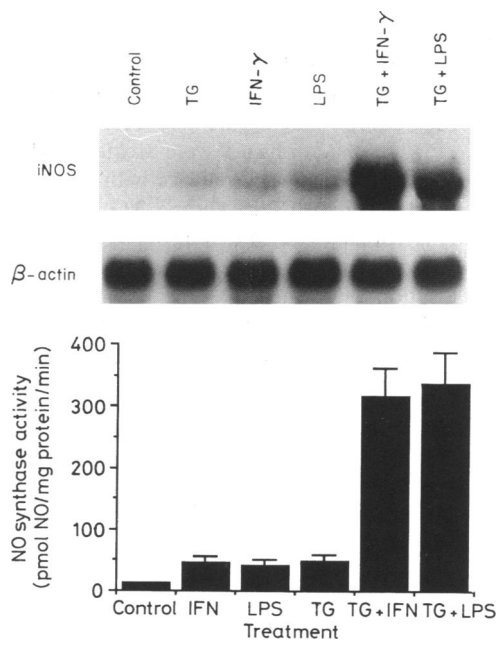

Figure 1. Induction of the expression of iNOS mRNA and iNOS activity by TG in combination with rIFN-γ or LPS. (a) TG plus rIFN-γ- or LPS-induced expression of iNOS mRNA. Thioglycollate-elicited peritoneal macrophages were plated at 2×10^7 cells/100-mm diameter Petri dishes. Then, the cells were treated with TG (300 nM), rIFN-γ (5 U/ml) or LPS (10 ng/ml) and cultured for 24 hr. Total RNA was prepared, and iNOS mRNA was analysed, by Northern hybridization as described in the Materials and Methods. The autoradiograms are shown. (b) TG plus rIFN-γ- or LPS-induced iNOS activity. Thioglycollate-elicited cells were treated with TG (300 nM), rIFN-γ (5 U/ml) or LPS (10 ng/ml) and cultured for 24 hr, harvested, sonicated and assayed for NOS activity. NO release was measured by using the method of Griess (nitrite). Results are presented as the means ± SD of triplicate cultures.

Effects of intracellular Ca^{2+} chelator BAPTA on the induction of NO synthesis

To confirm that iNOS induction was due to the increase in the intracellular Ca^{2+} level, we pretreated cells with BAPTA-AM to block the increase in cytosolic free Ca^{2+}. Macrophages were preincubated for 30 min in either medium alone or medium that contained 25 μM BAPTA-AM. Intracellular Ca^{2+} chelator BAPTA was generated by the intracellular esterases from BAPTA-AM added to the extracellular medium.[27,28] Then, BAPTA-loaded cells were stimulated with TG (300 nM) or rIFN-γ (5 U/ml) in combination with LPS (10 ng/ml) and cultured for 24 hr at 37°. Under these conditions the induction of NO production was completely inhibited (Fig. 2).

Intracellular Ca^{2+} as a 'priming' signal on the induction of NO synthesis

For full activation, macrophages require a primary signal for 'priming' and a second signal for 'triggering' in the induction of NO synthesis. Regulation of NO synthesis appears to be primarily at the level of transcription of the iNOS gene (Fig. 1a). To understand the effects of Ca^{2+} in synergistic co-operation

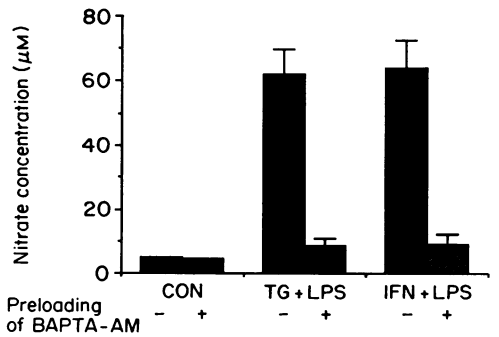

Figure 2. Effect of intracellular Ca^{2+} chelator BAPTA on the induction of NO synthesis in murine macrophages. Thioglycollate-elicited macrophages (2×10^5) were preincubated for 30 min in either medium alone or in a medium that contained 25 μM BAPTA-AM. Then, the cells were stimulated with TG (300 nM) in combination with rIFN-γ (5 U/ml) or LPS (10 ng/ml) and cultured for 48 hr. The amount of NO_2^- release was measured by using the method of Griess (nitrite). Data are presented as the means ± SD of triplicate cultures.

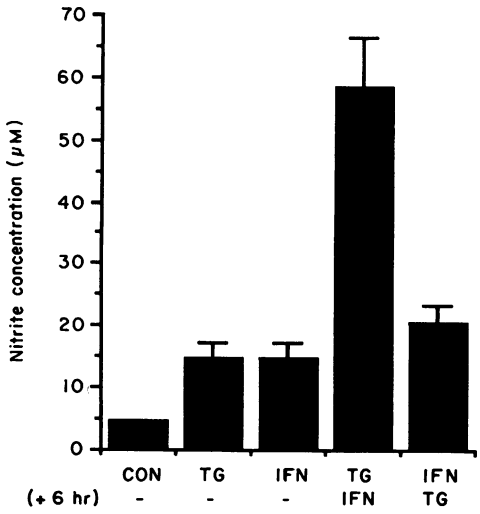

Figure 3. Pretreatment of rIFN-γ before stimulation with TG abolished the synergistic co-operation among TG and rIFN-γ. Thioglycollate-elicited macrophages (2×10^5) were cultured for 6 hr either in a medium alone or in a medium that contained TG (300 nM) or rIFN-γ (5 U/ml). Then, the cells were treated with TG or rIFN-γ as indicated. The amount of NO_2^- released was measured after 42 hr of incubation by using the method of Griess (nitrite). Values are the means ± SD of three experiments.

with IFN-γ or LPS on NO production, the effect of Ca^{2+} modulator on the NO production was analysed before and after the treatment of the cells with rIFN-γ. Figure 2 shows that TG did not increase the induction of NO production when the cells were treated with TG 6 hr after the treatment with rIFN-γ (Fig. 3). But, when the cells were treated with TG before or simultaneously with the treatment with rIFN-γ, TG showed synergistic action in combination with rIFN-γ (Fig. 3, Table 2).

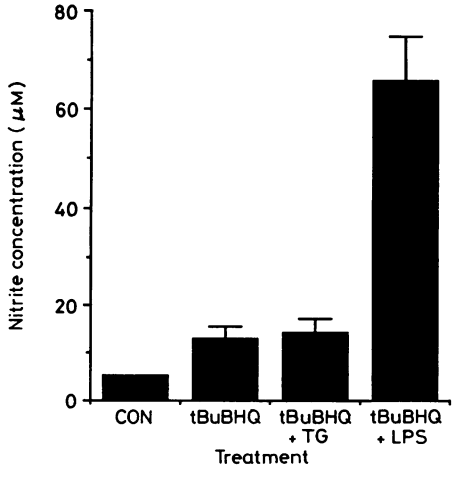

Figure 4. Effect of TG on the production of NO by tBuBHQ-stimulated macrophages. Thioglycollate-elicited macrophages (2×10^5) were cultured for 6 hr in the presence or absence of tBuBHQ (5 μM), washed and replaced with tBuBHQ-free medium, and stimulated with TG (300 nM) or rIFN-γ (5 U/ml). Then 42 hr later, supernatants were harvested and NO release was measured by using the method of Griess (nitrite). Results are presented as the means ± SD of three experiments.

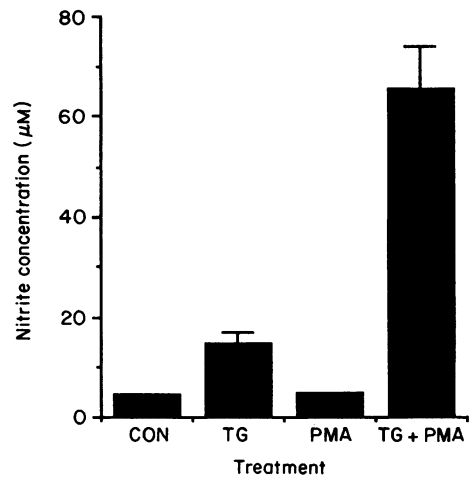

Figure 5. Effect of PMA on the production of NO by TG-stimulated macrophages. Thioglycollate-elicited macrophages (2×10^5) were cultured for 6 hr either in a medium alone or in a medium that contained TG (300 nM). Then, the cells were treated with PMA (200 nM), as indicated, and cultured for 42 hr. The amount of NO_2^- released was measured by using the method of Griess (nitrite). Data are presented as the means ± SD of three experiments.

In addition, we used another potent Ca^{2+} modulator, tBuBHQ, as synthetic compound which is chemically unrelated to TG. As shown in Table 2, the effect of tBuBHQ on NO production is similar to that of TG. But the effect of tBuBHQ can be abrogated by changing the media for a fresh one.[29] As shown in Fig. 4, macrophages were treated with tBuBHQ (5 μM), cultured for 6 hr, washed twice with fresh media, and stimulated with TG or LPS. Adding TG to the tBuBHQ-stimulated macrophages failed to induce NO synthesis. Finally, we demonstrated that, in cultured murine peritoneal macrophages, elevation of intracellular Ca^{2+} is sufficient for the expression of the iNOS gene. We previously reported that PKC activator, PMA, does not take part in the 'priming' signal, but exerts a 'triggering' signal in a synergistic co-operation.[14] As shown in Fig. 5, PMA (200 nM) alone had no effect on the production of NO. Interestingly, TG synergistically increased NO production when the cells were treated with PMA 6 hr after the treatment of TG. These results suggest that the role of intracellular Ca^{2+} acts as a 'priming' signal in the induction of NO synthesis rather than a 'triggering' signal.

DISCUSSION

Several recent reports demonstrate that activation of cultured macrophages requires two external signals such as IFN-γ and LPS for full activation, which are in turn controlled by the sequential exposure of responsive macrophages to 'priming' and 'triggering' signals.[7,16,30] Despite intensive research, the exact mechanisms of intracellular Ca^{2+} involved in the 'priming' and 'triggering' are understood only in part, thus it is necessary to elucidate the roles of Ca^{2+} in the regulatory mechanisms in the induction of NO synthesis. Intracellular Ca^{2+} signals have been implicated as potent regulators in the control of many aspects of cellular metabolism, including that of growth control regulation and tumorigenic transformation.[31,32]

One reagent commonly used to study the role of Ca^{2+} in the regulation of gene expression in mammalian cells is the calcium ionophore A23187, which is used extensively to mimic the effect of many physiological cell stimuli related to Ca^{2+}.[33–35] Calcium homeostasis is maintained by complex interactions among many signalling systems, and increases in intracellular Ca^{2+} concentration can be caused by complex mechanisms. An important issue is the nature of the intracellular pool from which Ca^{2+} is mobilized. The key to understanding these complex Ca^{2+} signals lies in understanding the interactions between the intracellular Ca^{2+} pool and the activities of the various Ca^{2+}-transporting systems that reverse the process. Thus, we examined the relationship between the induction of NO synthesis and intracellular Ca^{2+} homeostasis in murine peritoneal macrophages.

This report provides evidence that the Ca^{2+} pool content is linked to the induction of NO synthesis in murine peritoneal macrophages (C57BL/6). Previous studies have suggested a role of intracellular Ca^{2+} in the 'priming' of macrophages for tumoricidal activity by IFN-γ. The possible effect of inducing Ca^{2+} influx via the ionophore A23187 was therefore examined.[15,17] On the contrary, another report demonstrates that Ca^{2+} can replace LPS as a 'triggering' signal to increase NO synthesis in IFN-γ-primed macrophage.[36] We then approached the roles of intracellular Ca^{2+} pool for the induction of iNOS expression in peritoneal macrophages obtained from C57BL/6 mice. The role of Ca^{2+} in gene regulation addresses fundamental control mechanisms of signal transduction.

More recently, two putative inhibitors of ER Ca^{2+}-ATPase, TG and tBuBHQ have also been used.[25,26] These specific inhibitors have permitted us to analyse the relationship between Ca^{2+} and iNOS expression. Clearly, TG and tBuBHQ readily enter intact cells and release Ca^{2+} from the Ins(1,4,5)P_3-sensitive Ca^{2+} pool in several cell lines, without themselves causing accumulation of inositol phosphates.[37] Despite the absence of Ca^{2+} storage in the cells, cells still maintain resting cytosolic Ca^{2+} levels and retain normal morphology, viability, and metabolic activity for several days.[38] Thus TG and tBuBHQ may provide a valuable tool for the analysis of intracellular signalling mechanisms. *In vitro* exposure of macrophages to TG primed the cells for nitrite secretion, and when TG was used in combination with IFN-γ or LPS, there was a marked co-operative induction of NO synthesis. This increment of NO production was also reflected as an increased amount of iNOS mRNA, as determined by Northern blotting (Fig. 1a). These data suggest that the induction of NO synthesis by inhibitors of ER Ca^{2+}-ATPase may be explained as a result of transcriptional activation of the iNOS gene through a rapid and transient increase of intracellular Ca^{2+} in cultured murine peritoneal macrophages.

Furthermore, we found that intracellular Ca^{2+} acted as a 'priming' signal on the induction of NO synthesis rather than a 'triggering' signal. The synergy is dependent on the proper sequence of exposure. To understand better the effects of intracellular Ca^{2+} in synergistic co-operation with IFN-γ or LPS on NO production, the effects of Ca^{2+} modulator on the NO production were analysed by changing exposure times and by using another potent, reversible Ca^{2+}-ATPase inhibitor, tBuBHQ (Figs 3 and 4). In addition, we confirmed that elevation of intracellular Ca^{2+} is sufficient for the expression of NOS in cultured murine peritoneal macrophages. As reported previously, the PKC activator PMA, which down-regulates PKC activity, does not take part in the 'priming' signal, but has the effect of a 'triggering' signal in synergistic co-operation.[14] As shown in Fig. 5, TG synergistically increased NO production when the cells were treated with PMA 6 hr after the treatment of TG. As reported in this paper, we demonstrated the correlation between intracellular Ca^{2+} and iNOS expression. We believe that gene expression mediated by elevation of cytosolic Ca^{2+} is involved with some protein kinase. However, the respective correlation of protein kinase and elevation of intracellular Ca^{2+} in iNOS gene expression is still unknown.

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REFERENCES

1. Moncada S., Palmer R.M.J. & Higgs E.A. (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* **43,** 109.
2. Nathan C.F. & Hibbs J.B. Jr. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr Opin Immunol* **3,** 65.
3. Narumi S., Finke J.H. & Hamilton T.A. (1990) Interferon-γ and IL-2 synergize to induce selective monokine expression in murine peritoneal macrophages. *J Biol Chem* **265,** 7036.
4. Cox G.W., Melillo G., Chattopadhyay U., Mullet D., Fertel R.H. & Varesio L. (1992) Tumor necrosis factor-α-dependent production of reactive nitrogen intermediates mediates IFN-γ-plus IL-2-induced murine macrophage tumoricidal activity. *J Immunol* **149,** 3290.
5. Green S.J., Crawford R.M., Hockmeyer JT., Meltzer M.S. & Nacy C.A. (1990) *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN-γ-stimulated macrophages by induction of TNF-α. *J Immunol* **145,** 4290.
6. Stuehr D.J. & Marletta M.A. (1985) Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* LPS. *Proc Natl Acad Sci USA* **82,** 7738.
7. Stuehr D.J. & Marletta M.A. (1987) Synthesis of nitrite and nitrate in murine macrophage cell lines. *Cancer Res* **47,** 5590.
8. Celada A., Gray P.W., Rinderknecht F. & Schreiber R.D. (1984) Evidence for a gamma interferon receptor that regulates macrophage tumoricidal activity. *J Exp Med* **160,** 55.
9. Pace R.L., Russel S.W., Torres B.A., Johnson H.M. & Gray P.W. (1983) Recombinant mouse γ interferon induces the priming step in macrophage activation for tumor cell killing. *J Immunol* **130,** 2011.
10. Nacy C.A., Belosevic M., Meltzer M.S. & Hoover D.L. (1988) Interferon and nonviral pathogens. *Immunol Ser* **42,** 167.
11. Cho H.J., Xie Q.-W., Calaycay J. *et al.* (1992) Calmodulin is a subunit of nitric oxide synthase from macrophages. *J Exp Med* **176,** 599.
12. Xie Q.-W., Cho H.J., Calaycay J. *et al.* (1992) Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256,** 225.
13. Hortelano S., Genaro A.M. & Bosca L. (1993) Phorbol esters induce nitric oxide synthase and increase arginine influx in cultured peritoneal macrophages. *FEBS* **320,** 135.
14. Jun C.D., Choi B.M., Ryu H. *et al.* (1994) Synergistic cooperation between phorbol ester and IFN-γ for induction of nitric oxide synthesis in murine peritoneal macrophages. *J Immunol* **153,** 3684.

15. Buchmuller-Rouiller Y., Betz-Corradin S. & Mauel J. (1992) Differential effects of prostaglandins on macrophage activation induced by calcium ionophore A23187 or IFN-γ. *J Immunol* **148,** 1171.
16. Somers S.D., Weiel J.E., Hamilton T.A. & Adams D.O. (1986) Phorbol esters and calcium ionophore can prime murine peritoneal macrophages for tumor cells destruction. *J Immunol* **136,** 4199.
17. Drysdale B.-E., Yapundich R.A., Shin M.L. & Shin H.S. (1987) Lipopolysaccharide-mediated macrophage activation: the role of calcium in the generation of tumoricidal activity. *J Immunol* **138,** 951.
18. Hogan M.M. & Vogel S.N. (1987) Lipid A-associated proteins provide an alternate 'second signal' in the activation of recombinant interferon γ-primed C3H/HeJ macrophages to a fully tumoricidal state. *J Immunol* **139,** 3697.
19. Vodovotz Y., Kwon N.S., Pospischil M., Manning J., Paik J. & Nathan C. (1994) Inactivation of nitric oxide synthase after prolonged incubation of mouse macrophages with IFN-γ and bacterial lipopolysaccharide. *J Immunol* **152,** 4110.
20. Stuehr D.J., Cho H.J., Kwon N.S., Weise M.F. & Nathan C.F. (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci USA* **88,** 7773.
21. Green L.C., Wanger D.A., Glogowski J., Skipper P.L., Wishnok J.S. & Tannenbaum S.R. (1982) Analysis if nitrate, nitrite, and [^{15}N] nitrate in biologic fluids. *Anal Biochem* **126,** 131.
22. Maniatis T., Fritsch E.F. & Sambrook J. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Auffray C. & Rougenon F. (1980) Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur J Biol* **107,** 303.
24. Chirgwin J.M., Pryzbyla R.J., MacDonald R.J. & Rutter W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18,** 5294.
25. Llopis J., Chow S.B., Kass G.E.N., Gahm A. & Orrenius S. (1991) Comparison between the effects of the microsomal Ca^{2+}-translocase inhibitors thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone on cellular calcium fluxes. *Biochem J* **277,** 553.
26. Mason M.J., Garcia-Rodriguez C. & Grinstein S. (1991) Coupling between intracellular Ca^{2+} stores and the Ca^{2+} permeability of the plasma membrane. *J Biol Chem* **266,** 20856.
27. Michelangeli F., Ruiz M.-C., Fernandez E. & Ciarrocchi A. (1989) Role of Ca^{2+} in H^+ transport by rabbit gastric glands studied with A23187 and BAPTA, an incorporated Ca^{2+} chelator. *Biochim Biophys Acta* **983,** 82.
28. Schonthal A., Sugarman J., Brown J.H., Hanley M.R. & Feramisco J.R. (1991) Regulation of c-*fos* and c-*jun* protooncogene expression by the Ca^{2+}-ATPase inhibitor thapsigargin. *Proc Natl Acad Sci USA* **88,** 7096.
29. Short A.D., Bian J., Ghosh T.K., Waldron R.T., Rybak S.L. & Gill D.L. (1993) Intracellular Ca^{2+} pool content is linked to control of cell growth. *Proc Natl Acad Sci USA* **90,** 4986.
30. Oswald L.P., Afroun S., Bray D., Petit J.F. & Lemaire G. (1992) Low response of BALB/c macrophages to priming and activating signals. *J Leukocyte Biol* **52,** 315.
31. Campbell A.K. (1983) *Intracellular Calcium: Its Universal Role as Regulator.* John Wiley & Sons, Inc., New York.
32. Resendez E. Jr., Ting J., Kim K.S., Wooden S.K. & Lee A.S. (1986) Calcium ionophore A23187 as a regulator of gene expression in mammalian cells. *J Cell Biol* **103,** 2145.
33. Duffus J.H. & Patternson L.J. (1974) Control of cell division in yeast using ionophore A23187 with calcium and magnesium. *Nature* **251,** 626.
34. Keppens S., Vandenheede J.R. & Dewulf H. (1977) On the role of calcium as a second messenger in liver for the hormonally induced activation of glycogen phosphorylase. *Biochim Biophys Acta* **496,** 448.
35. Maino V.C., Green N.W. & Crumpton M.J. (1974) The role of calcium ions in initiating transformation of lymphocytes. *Nature* **251,** 324.
36. Raddassi K., Berthon B., Petit J.-F. & Lemaire G. (1994) Role of calcium in the activation of mouse peritoneal macrophages: induction of NO synthase by calcium ionophores and thapsigargin. *Cell Immunol* **153,** 443.
37. Jackson T.R., Patterson S.I., Thastrup O. & Hanley M.R. (1988) A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free Ca^{2+} without generation of inositol phosphates in NG115-401L neuronal cells. *Biochem J* **253,** 81.
38. Ghosh T.K., Bian J., Short A.D., Rybak S.L. & Gill D.L. (1991) Persistent intracellular calcium pool depletion by thapsigargin and its influence on cell growth. *J Biol Chem* **266,** 24690.