Bi-directional effects of the elevation of intracellular calcium on the expression of inducible nitric oxide synthase in J774 macrophages exposed to low and to high concentrations of endotoxin

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Nitric oxide produced through the action of inducible nitric oxide synthase (iNOS) is an important mediator in immune responses of the host. Various extracellular factors, including inflammatory stimuli, affect intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$), modulating cellular signalling and gene expression. In the present study we investigated the effects of increased $[Ca^{2+}]_i$ on NO production through the iNOS pathway in J774 macrophages. Thapsigargin (TG), a Ca^{2+}-ATPase inhibitor, and the Ca^{2+} ionophore A23187 were used as tools to induce an increase in $[Ca^{2+}]_i$ in the cytosol. This increase was confirmed by the fura 2 method. The production of NO was measured as accumulated nitrite in the cell culture medium; iNOS protein and iNOS mRNA were detected by Western blotting and reverse-transcriptase-mediated PCR respectively. The activation of nuclear factor κB (NF-κB) was investigated by electrophoretic mobility-shift assay. TG (100 nM) induced a marked synthesis of iNOS mRNA, iNOS protein and NO in cells primed with a low concentration of endotoxin [lipopolysaccharide (LPS) 1 ng/ml], which on its own induced barely detectable NO synthesis. Stimulation by a high concentration of LPS (100 ng/ml) induced a marked expression of iNOS and NO production. Under these conditions, treatment with TG hindered the synthesis of iNOS protein and NO production by accelerating the degradation of iNOS mRNA. Treatment with TG (100 nM) did not affect the NF-κB activity induced by low (1 ng/ml) or high (100 ng/ml) concentrations of LPS. Viability of the cells was confirmed by the 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2*H*-tetrazolium-5-carboxyaniline ('XTT') method; apoptosis was ruled out by propidium iodide staining and flow cytometry. A23187 (1 μM) also transiently increased $[Ca^{2+}]_i$ and had opposite effects on NO production depending on the LPS concentration. Our results show that increased $[Ca^{2+}]_i$ induced the stimulation or suppression of NO production through iNOS in macrophages depending on the state of cell activation. These findings suggest that the receptor-mediated increase in $[Ca^{2+}]_i$ might be an important factor in the control of the balance between the up-regulation and down-regulation of inflammatory genes, including that encoding iNOS, depending on the phase of the inflammatory response.

Key words: inflammation, host defence, mRNA stability, nuclear factor κB.

INTRODUCTION

Nitric oxide (NO), produced by three distinct isoforms of nitric oxide synthase (NOS) [1], regulates physiological and pathophysiological events in cellular systems. The induction of inducible nitric oxide synthase (iNOS) is involved in inflammatory processes evoked by endogenous and exogenous signals such as infectious agents, lipopolysaccharide (LPS) and proinflammatory cytokines in phagocytes and other cells [2].

In inflammation, NO produced by the action of iNOS functions as a non-selective killer mechanism against viruses and bacteria [3,4]. Excessive amounts of NO are also associated with plasma leakage and microvascular injury [5] as well as cytotoxic effects against host cells in the inflammatory focus. In septic shock, decreased blood pressure and organ failure are caused by the large amounts of NO produced by iNOS [6]. In addition, the reaction between NO and superoxide ($O_2^{-\cdot}$) produced by activated leucocytes that have migrated into the inflammatory focus results in the formation of the highly reactive and toxic compound peroxynitrite ($ONOO^-$), which is associated with the tissue injury seen at the site of inflammation [7,8].

Free intracellular Ca^{2+} is a pleiotropic regulatory substance in various types of cell; many of the effects of free intracellular Ca^{2+} involve the expression of new genes [9,10]. The activation of G-protein-linked cell-surface receptors by various extracellular signalling molecules induces the formation of Ins(1,4,5)P_3, which releases Ca^{2+} from intracellular stores. The depletion of Ca^{2+} from the endoplasmic reticulum provides a signal to the plasma membrane and opens the Ca^{2+} channels in the plasma membrane, causing an influx of Ca^{2+} ions into the cell. This event is called capacitative Ca^{2+} entry [11]. It has been shown that free intracellular Ca^{2+} participates in the induction of activation of nuclear factor κB (NF-κB) in human Jurkat T cells [12–14]. NF-κB is a key transcription factor in the regulation of expression of the gene encoding iNOS in various cell types [2,15]. There have been a few, controversial, observations on the role of Ca^{2+} in the

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; NO, nitric oxide; PDTC, pyrrolidine dithiocarbamate; RT–PCR, reverse-transcriptase-mediated PCR; TG, thapsigargin; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2*H*-tetrazolium-5-carboxyaniline.

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regulation of iNOS expression and NO synthesis, reporting either stimulatory [16–18] or inhibitory [19,20] effects.

The purpose of the study was to investigate the role of an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the regulation of iNOS expression and NO production by using the Ca^{2+}-ATPase inhibitor thapsigargin (TG) and the Ca^{2+} ionophore A23187 as tools to increase $[Ca^{2+}]_i$. The main finding was that $[Ca^{2+}]_i$ has bi-directional effects on NO production in a single cell type depending on the state of cell activation. In cells primed with low concentrations of LPS, the increase in $[Ca^{2+}]_i$ led to enhanced iNOS expression and NO production, whereas in cells treated with high concentrations of LPS, TG inhibited NO formation and decreased iNOS protein expression through destabilizing iNOS mRNA. Our findings suggest that Ca^{2+} might have a significant regulatory role in controlling the inflammatory process.

MATERIALS AND METHODS

Cell cultures and nitrite determination

J774 murine macrophages (A. T. C. C., Manassas, VA, U.S.A.) were cultured in 37 °C in a humidified air/CO_2 (19:1) atmosphere in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, penicillin (100 i.u./ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml), then harvested with trypsin/EDTA. Cells were seeded on 24-well plates and grown to confluence. Confluent cells were exposed to culture medium containing the compounds of interest. The culture medium was collected after 24 h incubations; nitrite, a stable product of NO in aqueous solutions, was measured with the Griess reaction as described [21].

Determination of cell viability and apoptosis

The number of viable macrophages was estimated by a colorimetric assay with the Cell Proliferation Kit II (Boehringer Mannheim, Germany). J774 macrophages were seeded on 96-well plates and incubated for 24 h at 37 °C in the presence of compounds of interest. The tetrazolium salt 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2*H*-tetrazolium-5-carboxyaniline (XTT), which is metabolized to formazan by intact mitochondrial dehydrogenases, and electron coupling reagent were added and cells were incubated for a further 4 h. The viability of cells was estimated on the basis of formazan formed, which was detected spectrophotometrically.

Macrophage apoptosis was determined by staining of DNA with propidium iodide and by flow cytometry (FACScan; Becton Dickinson, San Jose, CA, U.S.A.). In brief, the 200 ***g*** cell pellet was gently resuspended in hypotonic fluorochrome solution [25 μg/ml propidium iodide in 0.1 % sodium citrate/0.1 % (v/v) Triton X-100]. The tubes were left overnight in the dark at 4 °C before flow cytometric analysis. Cells showing a decreased relative DNA content were considered to be apoptotic.

Western blot analysis

Cells were seeded on six-well plates and grown to confluence, then stimulated for 12 h. Cell pellets from J774 cells were lysed in ice-cold extraction buffer [10 mM Tris base/5 mM EDTA/50 mM NaCl/1 % (v/v) Triton X-100/0.5 mM PMSF/2 mM Na_3VO_4/10 μg/ml leupeptin/25 μg/ml aprotinin/1.25 mM NaF/1 mM sodium pyrophosphate/10 mM n-octyl β-D-glucopyranoside, pH 7.4]. After extraction by incubation on ice for 15 min, samples were centrifuged and the resulting supernatant was boiled for 5 min in sample buffer [62.5 mM Tris/HCl/20 % (v/v) glycerol/2 % (w/v) SDS/10 mM 2-mercaptoethanol, pH 6.2] and stored at −70 °C until required. An aliquot of the supernatant was used to determine protein by the Coomassie Blue method [22]. Protein samples (20 μg) were separated by SDS/PAGE [10 % (w/v) gel] and transferred to nitrocellulose. iNOS protein was identified by Western blotting with rabbit polyclonal iNOS antibody obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Measurement of $[Ca^{2+}]_i$ and Ca^{2+} influx

Macrophages (5×10^7/ml) were loaded with the acetoxymethyl ester form of the fluorescent probe fura 2 (5 μM) for 30 min at 37 °C, in a shaking water-bath. Macrophages were diluted with 2 vol. of Hepes buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.5 mM Ca^{2+}, pH 7.40 at 37 °C, and kept at room temperature for 10 min to permit re-equilibration. Cells were washed twice and suspended in Hepes buffer to yield a cell suspension containing 5×10^6 macrophages/ml of buffer. The changes in fluorescence were recorded with a Shimadzu RF-5000 spectrofluorimeter (Shimadzu Corp., Kyoto, Japan) in thermostatically controlled (37 °C) quartz cuvettes, with continuous stirring. The excitation wavelengths were set at 340 and 380 nm when changes in $[Ca^{2+}]_i$ were measured.

Macrophages were incubated with the studied drug for 3 min before stimulation. Increases in $[Ca^{2+}]_i$ were stimulated by addition of TG (100 nM) or A23187 (1 μM). Calibration of the signal was performed with the method described by Grynkiewicz et al. [23] and Kankaanranta and Moilanen [24]. The maximal fluorescence (F_{max}) was measured after the addition of 2 μM ionomycin; the minimum fluorescence (F_{min}) was measured in the presence of 25 mM EGTA (pH 8.6)/0.1 % (v/v) Triton X-100. $[Ca^{2+}]_i$ values (nM) were calculated from the equation $[Ca^{2+}]_i = 224R(F-F_{min})/(F_{max}-F)$, where 224 is the dissociation constant for fura 2, F is the fluorescence of the intact cell suspension, and R is the ratio of F_{min} to F_{max} at 380 nm.

The resting $[Ca^{2+}]_i$ of macrophages suspended in Hepes was 114 ± 16 nM ($n = 10$). After stimulation with 100 nM TG and with 1 μM A23187, the maximal $[Ca^{2+}]_i$ values were 303 ± 61 nM (S.E.M., $n = 5$) and 261 ± 89 nM ($n = 5$) respectively.

RNA extraction and reverse-transcriptase-mediated PCR (RT–PCR)

Cells were grown on six-well plates to confluence, then stimulated for the durations indicated. Cells were trypsinized and washed twice with ice-cold PBS. Cell pellets were homogenized with a QIAshredder™ (Qiagen, Santa Clarita, CA, U.S.A.); extraction of total RNA was performed with an RNeasy® kit (Qiagen) for the isolation of total RNA. Synthesis of cDNA from mRNA and subsequent amplification of cDNA by PCR were performed with a GeneAmp® Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit (Perkin-Elmer; Roche Molecular Systems, Branchburg, NJ, U.S.A.). First-strand cDNA was synthesized with sequence-specific downstream primers for mouse iNOS or mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as a reference. The upstream primer was added to the reaction at the beginning of PCR amplification. PCR conditions were denaturation, annealing and extension at 94 °C for 35 s, 60 °C for 2 min and 72 °C for 2 min respectively, and 28 cycles for both iNOS and GAPDH. The concentration of Mg^{2+} in the PCR mixture was 1.5 mM. Primers (Pharmacia Biotech; Genosys

Biotechnologies, Cambridge, U.K.) for the amplification of GAPDH were 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ for downstream orientation and 5′-CGGAGTCAACGGCTTT-GGTCGTAT-3′ for upstream orientation, resulting in a 306 bp product [25]. Primers (Pharmacia Biotech; Genosys Biotechnologies) for the amplification of a cDNA sequence for murine iNOS mRNA were as follows: downstream primer, 5′-CACT-TCCTCCAGGATGTTGTA-3′, complementary to the sequence corresponding to bases 1137–1167; upstream primer, 5′-ATGC-CCGATGGCACCATCAGA-3′, corresponding to bases 796–816 in the murine iNOS mRNA and generating a fragment 372 bp in length [26,27]. Products were analysed on 2% (w/v) agarose gel containing ethidium bromide, then detected under UV.

Preparation of nuclear extracts

J774 macrophages were incubated for 30 min with the compounds being tested. Cells were then rapidly washed with ice-cold PBS and solubilized in hypotonic buffer A [10 mM Hepes/KOH (pH 7.9)/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM dithiothreitol/0.2 mM PMSF/10 μg/ml leupeptin/25 μg/ml aprotinin/0.1 mM EGTA/1 mM Na_3VO_4/1 mM NaF]. After incubation on ice for 10 min, cells were vortex-mixed for 30 s and the nuclei were separated by centrifugation at 15000 rev./min (21000 ***g***) and at 4 °C for 10 s. Nuclei were resuspended in buffer C [20 mM Hepes/KOH (pH 7.9)/25% (v/v) glycerol/420 mM NaCl/1.5 mM $MgCl_2$/0.2 mM EDTA/0.5 mM dithiothreitol/0.2 mM PMSF/10 μg/ml leupeptin/25 μg/ml aprotinin/0.1 mM EGTA/1 mM Na_3VO_4/1 mM NaF] and incubated on ice for 20 min. Nuclei were vortex-mixed for 30 s and nuclear extracts were obtained by centrifugation at 15000 rev./min (21000 ***g***) and 4 °C for 2 min. Protein content of the nuclear extracts was measured by the Coomassie Blue method [22].

Electrophoretic mobility-shift assay (EMSA)

Single-stranded oligonucleotides (5′-AGTTGAGGGGACTTT-CCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′) (Amersham Pharmacia Biotech) containing NF-κB-binding sequences were annealed and 5′-end-labelled with ^{32}P by using a DNA 5′-End Labeling Kit (Boehringer Mannheim). For binding reactions 10 μg of nuclear extract was incubated for 20 min at room temperature in a total reaction volume of 20 μl containing 0.1 mg/ml poly(dI-dC), 1 mM dithiothreitol, 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 200 mM KCl and 10% (v/v) glycerol. ^{32}P-labelled oligonucleotide probe (0.2 ng) was added to the reaction mixture and incubated for 10 min. Protein–DNA complexes were separated from the DNA probe by native PAGE [4% (w/v) gel]. The gel was dried and autoradiographed at −70 °C with an intensifying screen.

Statistics

Results are expressed as means ± S.E.M.; statistical significance was calculated by analysis of variance supported by Dunnett adjusted significance levels. Differences were considered significant at $P < 0.05$.

Materials

DMEM and its supplements were purchased from Gibco BRL (Paisley, Renfrewshire, U.K.). A23187, fura 2 acetoxymethyl ester, TG, ionomycin, EGTA, bacterial LPS (*Escherichia coli* 0111:B4), cycloheximide, actinomycin D, ethidium bromide, Tris base, EDTA, NaCl, Triton X-100, PMSF, Na_3VO_4, leupeptin, aprotinin, NaF, sodium pyrophosphate, n-octyl β-D-glucopyranoside, Tris/HCl, glycerol, SDS, 2-mercaptoethanol, sulphanilamine and naphthylethylenediamine dihydrochloride were from Sigma (St Louis, MO, U.S.A.). Agarose (Promega Corp., Madison, WI, U.S.A.), N^G-Monomethyl-L-arginine (L-NMMA; Clinalfa, Läufelfingen, Switzerland), L-*N*-iminoethyl-ornithine (Alexis Corp., Läufelfingen, Switzerland), pyrrolidine dithiocarbamate (PDTC; Tocris, Langford, Bristol, U.K.), and Ro 31-8220 (Roche Products Ltd, Welwyn Garden City, Herts., U.K.) were obtained as indicated.

RESULTS

Bi-directional effects of TG and A23187 on LPS-induced production of NO in J774 cells

J774 murine macrophages produced NO, measured as the accumulation of nitrite in the culture medium, in response to LPS during a 24 h incubation; the amount of nitrite produced was dependent on the concentration of LPS added. An LPS concentration of 1 ng/ml caused a barely detectable synthesis of NO in J774 cells and at 100 ng/ml it induced a marked formation of NO, as shown in Figure 1. LPS-induced nitrite accumulation was totally inhibited by the NOS inhibitors L-*N*-iminoethyl-ornithine and L-NMMA, each at 1 mM, indicating that it was due to NO production (results not shown).

TG, a sesquiterpene lactone, was used as a pharmacological tool to mimic the receptor-mediated Ca^{2+} signalling [11,28] on LPS-induced NO production in J774 macrophages. The macrophages failed to produce NO in response to TG (10–100 nM) in the absence of LPS; however, 100 nM TG significantly enhanced

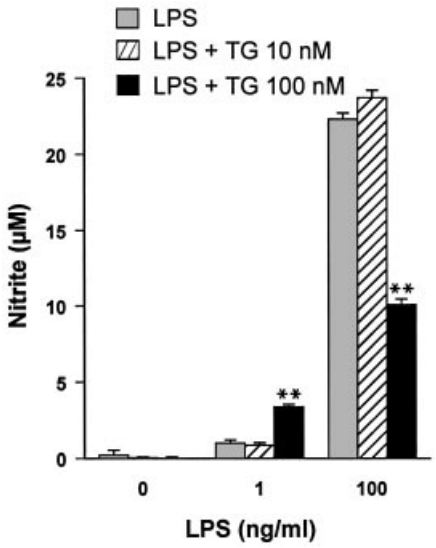

Figure 1 Effects of combination of TG and LPS on nitrite production in J774 macrophages

Cells were treated with LPS (0–100 ng/ml) and TG for 24 h; nitrite levels were determined in the culture medium. Results are expressed as means ± S.E.M. ($n = 6$). $^{**}P < 0.01$ compared with cells treated with LPS (1–100 ng/ml) only.

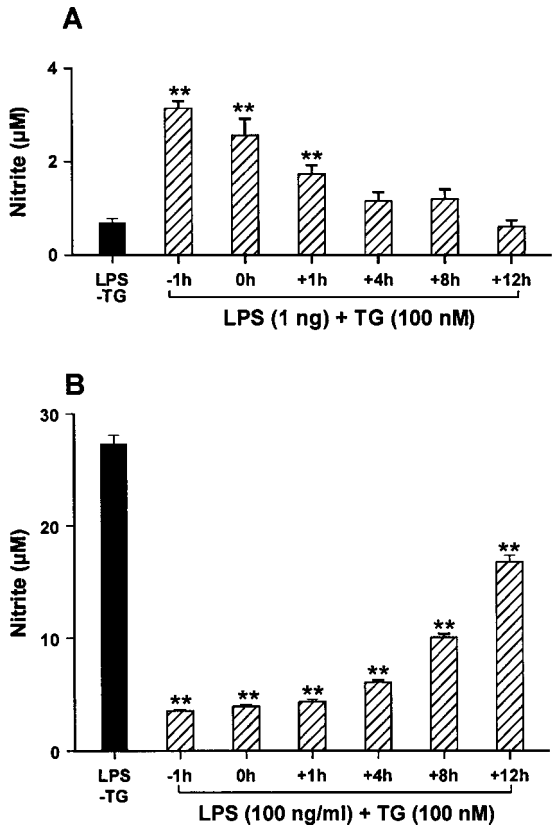

Figure 2 Time-dependent effect of TG challenge on nitrite production in J774 macrophages incubated with a low (1 ng/ml) (A) or high (100 ng/ml) (B) concentration of LPS

Cells were treated with 1 ng/ml LPS (**A**) and with 100 ng/ml LPS (**B**); 100 nM TG was added to the cells at the time points indicated. Cells were incubated for 24 h, after which nitrite levels were determined in the culture medium. Results are expressed as means ± S.E.M. ($n = 6$). $^{**}P < 0.01$ compared with cells treated with LPS (1 or 100 ng/ml, as appropriate) only.

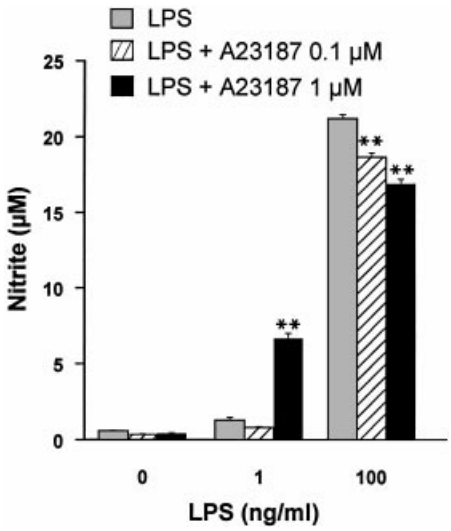

Figure 3 Effects of a combination of A23187 and LPS on nitrite production in J774 macrophages

Cells were treated with LPS (0–100 ng/ml) and A23187 (0.1–1 μM) for 24 h, after which nitrite levels were determined in the culture medium. Results are expressed as means ± S.E.M. ($n = 6$). $^{**}P < 0.01$ compared with cells treated with LPS only.

Table 1 Effect of thapsigargin (100 nM) on $[Ca^{2+}]_i$ in J774 macrophages treated with LPS

$[Ca^{2+}]_i$ was measured by fura-2-labelled macrophages before treatment (basal), after $[Ca^{2+}]_i$ had reached its maximum (peak) and 2 min after challenge with TG (sustained). Results are means ± S.E.M. ($n = 5$).

	$[Ca^{2+}]_i$ (nM)		
Treatment	Basal	Peak	Sustained
TG (100 nM)	104 ± 21	303 ± 61	190 ± 31
LPS (1 ng/ml) + TG (100 nM)	111 ± 23	326 ± 41	173 ± 25
LPS (100 ng/ml) + TG (100 nM)	121 ± 20	330 ± 62	187 ± 31

NO formation in cells primed with a low concentration of LPS (1 ng/ml) that on its own induced barely detectable NO synthesis (Figure 1). In contrast, NO formation was inhibited by 100 nM TG in cells that were stimulated with higher concentrations of LPS (100 ng/ml) to produce enhanced amounts of NO (Figure 1). These results suggest that a Ca^{2+} signal potentiated NO production in macrophages primed with low concentrations of LPS and inhibited the high-output synthesis of NO induced by higher concentrations of LPS. Figure 2 shows the time courses of the stimulatory and inhibitory actions of TG on the production of NO. The stimulatory action in the presence of low concentrations of LPS was found when TG was added 1 h before, at the same time as, and 1 h after LPS. If TG was added 4–12 h after LPS no effect on NO production was found (Figure 2A). The inhibitory action of TG on NO production induced by a high concentration of LPS was most marked when TG was added 1 h before or at the same time as LPS and declined gradually thereafter. However, TG added 12 h after LPS still caused a 40% inhibition (Figure 2B). These results show that the time course of the stimulatory and inhibitory effects were different and suggest that the two actions had distinct mechanisms.

The viability of the cells after experiments performed for nitrite determination was confirmed by the XTT cytotoxicity assay; apoptosis was measured by a flow-cytometric analysis of the relative DNA content in cells stained with propidium iodide. In cells exposed to LPS or TG or both, no marked cytotoxicity or apoptosis was detected.

The findings on the bi-directional effect of $[Ca^{2+}]_i$ were supported by results from studies with A23187. In a comparable manner to the action of TG, A23187 (1 μM) did not induce a detectable NO release in the absence of LPS and caused a statistically significant increase in NO production in cells primed with a low concentration (1 ng/ml) of LPS (Figure 3). When J774 cells were stimulated with higher concentrations of LPS (100 ng/ml) to produce enhanced amounts of NO, A23187 suppressed NO synthesis (Figure 3).

Effects of TG and A23187 on $[Ca^{2+}]_i$ in LPS-stimulated J774 cells

Changes in $[Ca^{2+}]_i$ were detected by the fura 2 method to investigate whether the changes in NO production with different combinations of LPS and agents affecting $[Ca^{2+}]_i$ homoeostasis

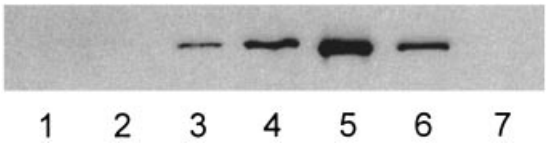

Figure 4 Effects of TG on iNOS protein expression in LPS-treated J774 macrophages

Cells were incubated with LPS alone or with LPS plus TG for 12 h, then harvested for Western blot analysis. Untreated cells (control, lane 1) were used as negative control. Lane 2, 100 nM TG; lane 3, 1 ng/ml LPS; lane 4, 1 ng/ml LPS plus 100 nM TG; lane 5, 100 ng/ml LPS; lane 6, 100 ng/ml LPS plus 100 nM TG; lane 7, 1 ng/ml LPS plus 100 nM TG plus 100 μM PDTC. The figure is representative of five separate experiments with similar results.

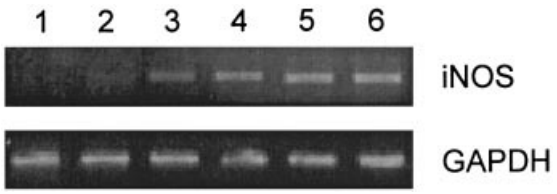

Figure 5 Effect of TG on iNOS mRNA accumulation in LPS-treated J774 macrophages

Cells were exposed to LPS alone or to LPS plus TG for 6 h, then harvested for extraction of total RNA. iNOS mRNA and GAPDH mRNA were detected by RT–PCR. Untreated cells (control, lane 1) were used as negative control. Lane 2, 100 nM TG; lane 3, 1 ng/ml LPS; lane 4, 1 ng/ml LPS plus 100 nM TG; lane 5, 100 ng/ml LPS; lane 6, 100 ng/ml LPS plus 100 nM TG. The figure is representative of four separate experiments with similar results.

could be explained on the basis of variations in Ca^{2+} influx and $[Ca^{2+}]_i$. LPS (1–100 ng/ml) did not evoke any detectable change in $[Ca^{2+}]_i$ in J774 cells. TG, when introduced to J774 macrophages, induced a rapid and transient influx of Ca^{2+} ions into the cytosol in a biphasic manner. TG caused a rapid increase in $[Ca^{2+}]_i$ followed by a sustained elevation of $[Ca^{2+}]_i$ that lasted several minutes. There were no differences in the magnitude or the shape of the Ca^{2+} flow induced by TG between control cells and cells treated with LPS (1–100 ng/ml), suggesting that LPS did not modulate TG-induced Ca^{2+} signalling (Table 1). Introduction of A23187 to the cells caused a rapid increase in $[Ca^{2+}]_i$ (basal 154 ± 23, peak 261 ± 89; $n = 5$) whose shape and magnitude were identical regardless of the presence of LPS (1 or 100 ng/ml).

Pharmacological control of TG-induced NO production in J774 cells primed with low concentrations of LPS

The L-arginine analogue L-NMMA, an inhibitor of NOSs (1 mM), and cycloheximide, a protein synthesis inhibitor (1 μg/ml), each abrogated (over 95% inhibition; $P < 0.01$, $n = 6$) TG-induced nitrite accumulation in cultures of J774 cells primed with low concentrations of LPS (1 ng/ml), indicating that the nitrite was due to the action of NOS and that protein synthesis *de novo* was essential. An inhibitor of NF-κB, PDTC (100 μM), hindered the TG-induced formation of NO (over 95% inhibition; $P < 0.01$, $n = 6$), suggesting that the activation of NF-κB is involved in TG-induced NO synthesis in LPS-primed

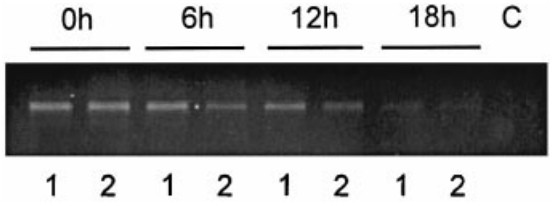

Figure 6 Effect of TG on the rate of degradation of iNOS mRNA in J774 macrophages

Cells were stimulated with 100 ng/ml LPS (lanes 1) solely or with a combination of 100 ng/ml LPS and 100 nM TG (lanes 2). After 6 h of incubation, actinomycin D (0.5 μg/ml) was added to the cells and incubated for the further duration indicated. After incubation, cells were harvested for extraction of total RNA; iNOS mRNA was detected by RT–PCR. Untreated cells (C) were used as a negative control. The figure is representative of three separate experiments with similar results.

macrophages. The protein kinase C inhibitor Ro 31-8220 (1 μM) [29] had no significant effect on TG-induced NO production.

Effects of TG on iNOS protein expression

The iNOS protein expression after challenge by LPS alone or in combination with TG was investigated by Western blotting. Unstimulated J774 macrophages and cells treated with 100 nM TG failed to show the induction of any detectable amounts of iNOS protein (Figure 4). Cells stimulated by low concentrations of LPS (1 ng/ml) expressed small amounts of iNOS protein; this expression was significantly enhanced by 100 nM TG (Figure 4). Cells stimulated with higher concentrations of LPS (100 ng/ml) expressed enhanced amounts of iNOS; this was decreased considerably by TG (100 nM). These bi-directional effects of TG on iNOS protein expression, depending on the concentration of LPS used as a stimulus, were consistent with the results gained from nitrite assays (see above). Treatment of J774 cells with PDTC hindered the expression of iNOS protein when it was induced by high concentrations of LPS (results not shown) or when cells were stimulated with a combination of LPS at low concentration and TG, indicating the crucial involvement of the activation of NF-κB in iNOS expression (Figure 4).

Effects of TG on iNOS mRNA expression

Unstimulated J774 macrophages and cells incubated in the presence of TG (100 nM) alone did not express detectable amounts of iNOS mRNA as assessed by RT–PCR (Figure 5). Macrophages treated with a low concentration of LPS (1 ng/ml) expressed small amounts of iNOS mRNA; this was enhanced by the addition of TG (100 nM) (Figure 5). When cells were stimulated with higher concentrations of LPS (100 ng/ml) no differences in the iNOS mRNA were found by this method between cells treated with TG (100 nM) and those that were not (Figure 5). However, in the latter case TG (100 nM) decreased the LPS-induced production of NO and expression of iNOS protein (Figures 1 and 4).

The hypothesis of repression of iNOS mRNA stability by $[Ca^{2+}]_i$ was tested by adding actinomycin D (0.5 μg/ml), an inhibitor of transcription, to cells after 6 h of incubation with LPS (100 ng/ml) either alone or in combination with TG (100 nM). The amount of iNOS mRNA was found to decrease faster in cells treated with TG and LPS than with LPS alone. The difference between iNOS mRNA levels could be observed at 6 h

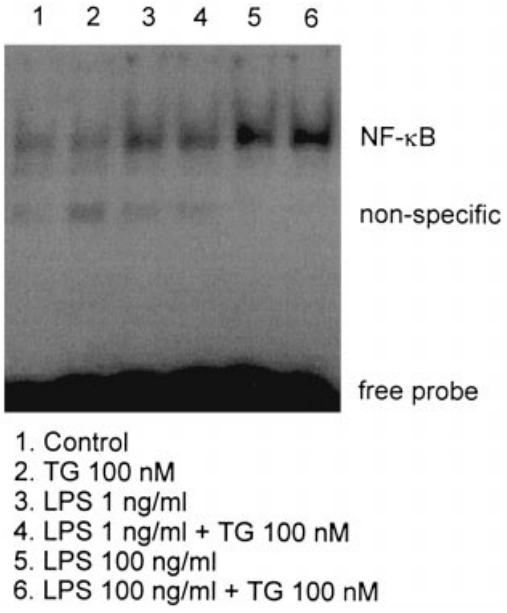

Figure 7 Activation of NF-κB following TG and LPS treatments in J774 macrophages

Cells were incubated with LPS alone or with LPS plus TG for 30 min before nuclear extracts were prepared and EMSA was performed. NF-κB is indicated; 'non-specific' means non-specific binding. Lane 1, untreated cells (control); lane 2, 100 nM TG; lane 3, 1 ng/ml LPS; lane 4, 1 ng/ml LPS plus 100 nM TG; lane 5, 100 ng/ml LPS; lane 6, 100 ng/ml LPS plus 100 nM TG. The figure is representative of four separate experiments with similar results.

and was marked at 12 h after the addition of actinomycin D (Figure 6).

Effects of TG on activation of NF-κB

TG-induced NO synthesis was inhibitable with the NF-κB inhibitor PDTC; increased $[Ca^{2+}]_i$ has been reported to be involved in NF-κB activation in T-cells [12,14]. We therefore measured the effects of TG on NF-κB activation by EMSA. Untreated control cells showed some basal NF-κB activity. LPS induced NF-κB activation in a concentration-dependent manner (Figure 7). TG alone did not enhance NF-κB activation; when added together with low (1 ng/ml) or higher (100 ng/ml) concentrations of LPS, TG did not alter NF-κB activation (Figure 7).

DISCUSSION

The present study shows that $[Ca^{2+}]_i$ has two distinct effects on NO production in J774 macrophages depending on the level of the stimulation of the cells. Increased $[Ca^{2+}]_i$ led to increased expression of iNOS and production of NO in cells primed with low concentrations of LPS; in cells treated with high LPS concentrations, increased $[Ca^{2+}]_i$ suppressed iNOS expression and NO production by a mechanism that resulted in accelerated degradation of iNOS mRNA.

Several extracellular mediators activate cells through G-protein coupled receptors. Receptor activation leads to the formation of Ins(1,4,5)P_3 and to an increase in $[Ca^{2+}]_i$; Ca^{2+} participates in the regulation of gene expression [10,11]. As tools to induce an increase in $[Ca^{2+}]_i$, TG mimicking receptor-mediated, i.e. capacitative, Ca^{2+} entry [11,28] and A23187 were used. TG and A23187 alone had no effect on NO production in macrophages but cells cultured in the presence of a concentration of LPS that alone induced barely detectable NO production responded to TG or A23187 by enhanced NO formation. There are observations suggesting that the increase in $[Ca^{2+}]_i$ is involved in the potentiation of NO production through the iNOS pathway in peritoneal macrophages [30] and in LPS-treated macrophages [17,18]. To provide support to the hypothesis that the NO measured was derived from the action of synthesized iNOS *de novo*, the concentrations of iNOS protein and mRNA were measured. These findings were consistent with the results on NO production: TG caused an enhanced accumulation of iNOS mRNA and protein in cells treated with low concentrations of LPS, whereas TG suppressed iNOS protein expression when given to cells treated with higher levels of LPS.

In characterizing the pathway resulting in the augmentation of NO production by TG in cells primed with low levels of LPS, some pharmacological tools were exploited. Nitrite production was hindered by the NOS inhibitor L-NMMA and by the protein synthesis inhibitor cycloheximide, indicating that the nitrite measured was derived from NO produced by the iNOS pathway.

TG-induced NO production in LPS-primed cells was insensitive to the protein kinase C inhibitor Ro 31-8220 but was totally inhibited by the NF-κB inhibitor PDTC. NF-κB activation is critical for the induction of iNOS transcription after challenge with LPS [15]. NF-κB is also sensitive to Ca^{2+} in T-cells [12,14] and NF-κB activation increases after a stimulus leading to the formation of Ins(1,4,5)P_3 [17], which classically elevates $[Ca^{2+}]_i$ levels. We therefore tested the effects of TG on NF-κB activation. TG alone had no effect on NF-κB activity as measured by EMSA. TG did not alter the NF-κB activation induced by a low or a high concentration of LPS. These results support the crucial role of NF-κB activation for the induction of iNOS and the proposal that TG regulates iNOS expression in an NF-κB-independent manner. Some other inflammatory transcription factors have been reported to be regulated by Ca^{2+}, i.e. NF-AT, Oct/OAP and activator protein 1 [31–33]. Of these, activator protein 1 has been shown to be involved in the regulation of human gene encoding iNOS in some cell types [34] and could therefore be a potential target of the stimulatory action of TG on iNOS induction in LPS-primed macrophages.

In fura 2 fluorescence measurements there were no significant differences in the shape or magnitude of Ca^{2+} influx curves into the cytosol in resting cells in comparison with cells treated with high or low levels of LPS in response to TG or A23187. This result suggests that the effect on iNOS expression was not due to an altered Ca^{2+} signal in response to TG or A23187 in cells treated with high or low levels of LPS and that an increase in $[Ca^{2+}]_i$ is not a sufficient signal to induce iNOS.

With the increased concentration of LPS, the exposure of the cells to TG caused an inhibition of iNOS expression and NO production. Further studies were designed to test two mechanisms that might mediate that effect, namely the effect of TG on the activation of NF-κB and on the stability of iNOS mRNA. A high concentration of LPS caused a marked activation of NF-κB but TG had no effect on NF-κB activation in cells challenged with LPS. Studies on mRNA stability indicated that iNOS mRNA was degraded more rapidly in cells treated with a combination of LPS and TG at high concentrations than in cells treated with LPS alone. The involvement of the inhibitory action of elevated $[Ca^{2+}]_i$ on NO production via the iNOS pathway is poorly known in the literature. Elevated $[Ca^{2+}]_i$ has been shown to inhibit cytokine-induced and LPS-induced iNOS-dependent NO production in human chondrocytes [19] and murine brain

microvascular endothelial cells [20]. Consistent with the present results is the observation that an increase in $[Ca^{2+}]_i$ in the cytosol decreases the stability of iNOS mRNA in chondrocytes, thereby shortening the half-life of iNOS mRNA and leading to a decrease in iNOS protein level and the production of NO [19]. The untranslated region at the 3′ end of the mRNA of several transiently expressed genes, including cytokines and human and murine iNOS, contains an AU-rich element ('ARE') that serves as a target for selective protein-mediated mRNA degradation [35–38]. It remains to be clarified whether these mechanisms are sensitive to Ca^{2+}.

Mechanisms controlling NO production in the different stages of inflammation, increasing at the beginning and decreasing at advanced stages, might be important. It is a tempting idea that at the beginning of the inflammatory response provoked by infectious agents (i.e. when levels of inflammatory products such as bacterial endotoxin are still low), various inflammatory mediators known to enhance receptor-activated Ca^{2+} entry result in enhanced NO formation through the iNOS pathway. Increased local NO production serves as a method of eliminating infective agents because it functions as a killing mechanism against pathogens [2–4]. Interestingly, it has recently been found that a toxin, α-haemolysin, derived from a pathogenic *E. coli* strain causes $[Ca^{2+}]_i$ oscillations and induces the production of pro-inflammatory cytokines in renal epithelial cells [39]. However, excessive amounts of NO derived from uncontrolled iNOS activity at the advanced stage of inflammation lead to an inhibition of mitochondrial respiration [40], DNA damage [41] and tissue injury [7,8], which might be detrimental to the host. Therefore, in the advanced stage of inflammation, when most of the bacteria have already been killed and endotoxin levels are high, Ca^{2+} signalling might inhibit NO production and thereby participate in the suppression of the inappropriate inflammatory response. On the basis of the present results we suggest that mediators affecting Ca^{2+} signalling similarly to TG (i.e. various G-protein-linked receptor-mediated signals) provide a possible pathophysiological mechanism regulating NO production in macrophages, depending on the stage of inflammation.

In conclusion, the present study suggests that increased intracellular Ca^{2+} levels have two separate effects on the expression of iNOS and NO production in J774 macrophages. In cells primed by a low LPS concentration, an increase in $[Ca^{2+}]_i$ potentiates NO production by enhancing the expression of iNOS in an NF-κB independent manner. An increase in $[Ca^{2+}]_i$ together with a high LPS concentration results in a decrease in the amount of iNOS protein and an inhibition of NO formation that is related to the decrease in iNOS mRNA stability.

We thank Mrs Niina Ikonen and Mrs Heli Määttä for excellent technical assistance. This study was supported by grants from the Medical Research Fund of Tampere University Hospital, the Foundation of Nutrition Research, Finland, and Tampere Tuberculosis Foundation.

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Received 24 August 2000/10 November 2000; accepted 11 December 2000