MODULATOR OF INTRACELLULAR Ca²⁺, THAPSIGARGIN, INTERFERES WITH *IN VITRO* SECRETION OF CYTOKINES AND NITRIC OXIDE

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Interference of thapsigargin (TG), an inhibitor of endoplasmic reticulum Ca²⁺ ATPase, with immune reactivity of murine macrophages was investigated under conditions *in vitro*. The activation of cells with lipopolysaccharide (LPS), interferon-γ (IFN-γ), and with acyclic nucleoside phosphonate N^6-isobutyl-9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (N^6-isobutyl-PMEDAP) resulted in enhanced production of cytokines TNF-α, IL-10, chemokines RANTES/CCL5 and MIP-1α/CCL3, as well as in substantially augmented production of nitric oxide (NO) triggered by IFN-γ. The effects were in a dual mode of action influenced by TG (1 μM). While TG upregulated secretion of TNF-α, it inhibited secretion of IL-10 and RANTES. The immune-stimulated secretion of MIP-1α remained virtually unaffected, though TG on its own activated expression of MIP-1α in macrophages. The high-output NO production induced by IFN-γ, high concentrations of LPS, or by combination of IFN-γ plus LPS or N^6-isobutyl-PMEDAP was inhibited by TG. On the other hand, production of NO which was marginally activated by low concentration of LPS was upregulated by TG.

INTRODUCTION

Physiologically maintained level of cytosolic free Ca²⁺ is a crucial signal controlling many cell functions[1,2], and any imbalance may have cytotoxic consequences[3]. Also the role of calcium ions in the expression of immunomodulatory properties of drugs and their importance in effectiveness of natural defence mechanisms are being intensively explored. A plausible experimental approach to study this problem is the use of modulators of intracellular calcium. Thapsigargin (TG) is a naturally occurring sesquiterpene lactone with prominent acute action on various types of cells. It is widely employed as a selective and irreversible inhibitor of endoplasmic reticulum Ca²⁺ ATPase leading to rapid elevation of intracellular concentration of free calcium. The interference of TG with immune functions of cells is largely unknown. We investigated its effects on production of cytokines including chemokines RANTES and MIP-1α under conditions *in vitro*.

MATERIALS AND METHODS

Animals and isolation of peritoneal macrophages

Female mice of the inbred strain C57BL/6, 7–9 wks old, were purchased from Charles River Deutschland (Sulzfeld, Germany). They were kept in transparent plastic cages in groups of eight, and maintained in an Independent Environmental Air Flow Cabinet (ESI Flufrance, Wissous, France). Lighting was set on 0600 to 1800 h, temperature at 22 °C.

Mice, sacrificed by cervical dislocation, were injected intraparitoneally with 8 ml sterile saline. Collected and pooled lavage cells were washed, resuspended in culture medium and seeded into 96-well flat-bottom Nunc microplates in 100-μl volumes. Adherent peritoneal cells (macrophages) were isolated by incubating the cells for 2 h at 37 °C, 5% CO_2, and then thrice vigorously shaking the plate and washing to remove non-adherent cells. They were cultured with or without addition of immunostimulatory agents, i.e interferon-γ (IFN-γ; 5000 pg/ml), lipopolysaccharide (LPS; 10-10.000 pg/ml), and N^6-isobutyl-9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (N^6-isobutyl-PMEDAP; 2-100 μM), and in the absence or presence of thapsigargin (TG; 1 μM) in Heraeus incubator for 24 h. TG was added 30 min before the immunostimulators. All experimental variants were set in duplicate.

Culture medium and chemicals

Complete RPMI-1640 medium contained 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μg/ml gentamicin, and 5×10^{-5} M 2-mercaptoethanol (all Sigma). Recombinant mouse IFN-γ was purchased from R&D Systems (Minneapolis, MN), LPS of S. typhimurium origin from Difco Labs (Detroit, MI). Acyclic nucleoside phosphonate N^6-isobutyl-9-[2-
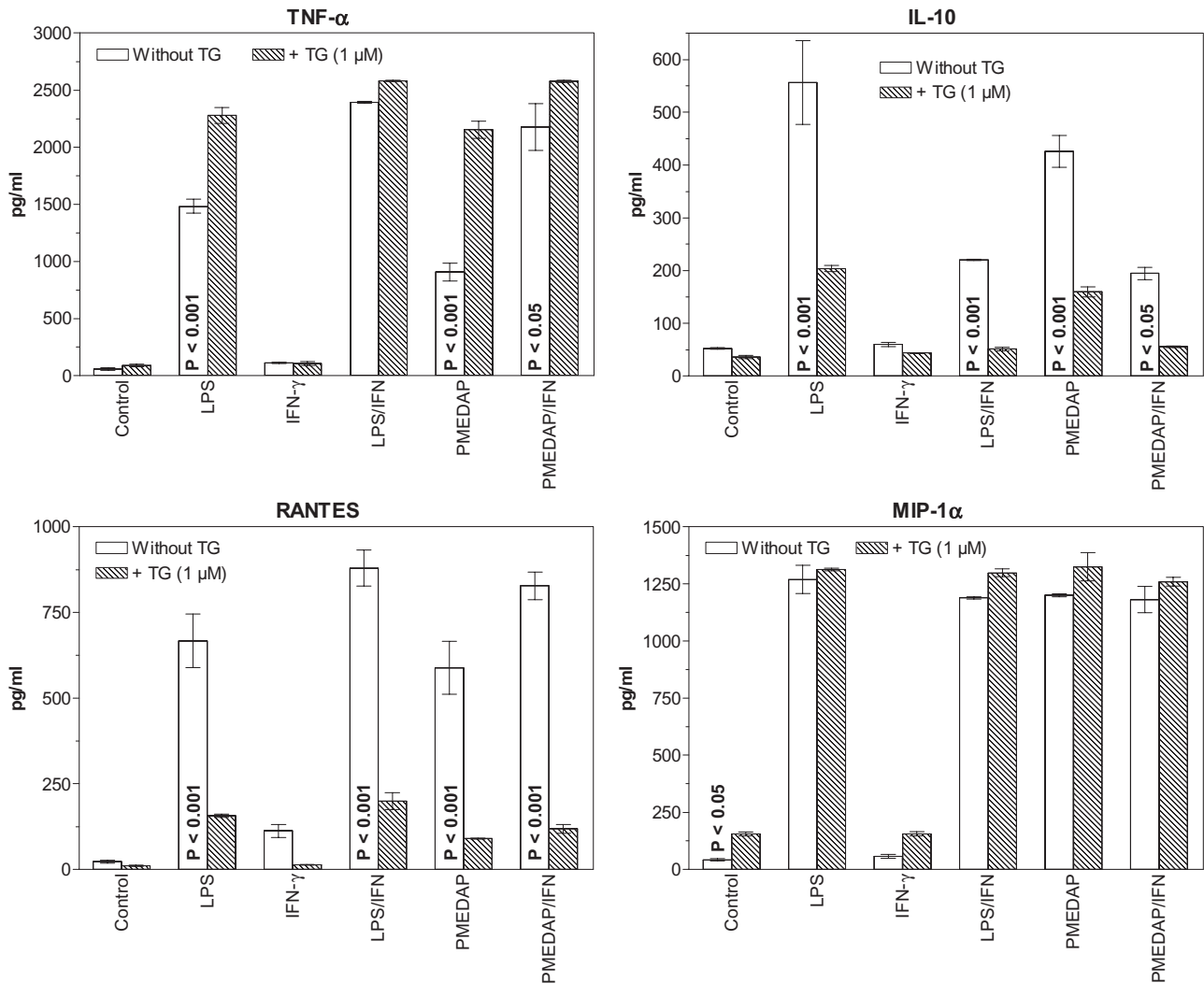
Fig. 1. Effect of thapsigargin (TG, 1 µM) on 5-h secretion of cytokines by mouse macrophages. The following concentrations of primary immune stimulators were applied: LPS 1 ng/ml, IFN-γ 5 ng/ml, N^6-isobutyl-PMEDAP (PMEDAP) 50 µM. The data are means ± SEM.

(phosphonomethoxy)ethyl]-2,6-diaminopurine (N^6-isobutyl-PMEDAP) was synthesized in-house (Inst. Organic Chemistry and Biochemistry) according to the procedure described elsewhere[4]. The cell viability and cell proliferation assay kit WST-1 was bought from Roche Applied Science (Mannheim, Germany). Thapsigargin was obtained from Sigma-Aldrich (Prague, CZ).

Immunobiological assays

Concentration of cytokines and chemokines in the 5-h cell supernatants was determined using enzyme-linked immunoabsorbent assay (ELISA) kits, following the manufacturer's instructions (R&D Systems, Minneapolis, MN).

The concentration of nitrites in the 24-h supernatants was taken as a measure of NO production. It was detected in individual cell-free samples (50 µl) incubated for 10 min at 37 °C with an aliquot of a Griess reagent (1% sulphanilamide/0.1% naphtylendiamine/2.5% H_3PO_4). The absorbance at 540 nm was recorded using a microplate spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert absorbance to µM nitrite.

Viability of macrophages was assayed after the 20 h of culture, following the manufacturer's instructions (Roche). Absorbances at 450 nm were determined at the interval of 2.5 h after addition of WST-1.

Data analysis

Analysis of variance (ANOVA) with subsequent Bonferroni's multiple comparison test for selected pairs, and graphical presentation of data were done using the Prism program (GrapPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Irrespective of the type of primary immune stimulation of macrophages, TG significantly augmented secretion of proinflammatory cytokine TNF-α (Fig. 1). This is in good line with the observation that transient increase
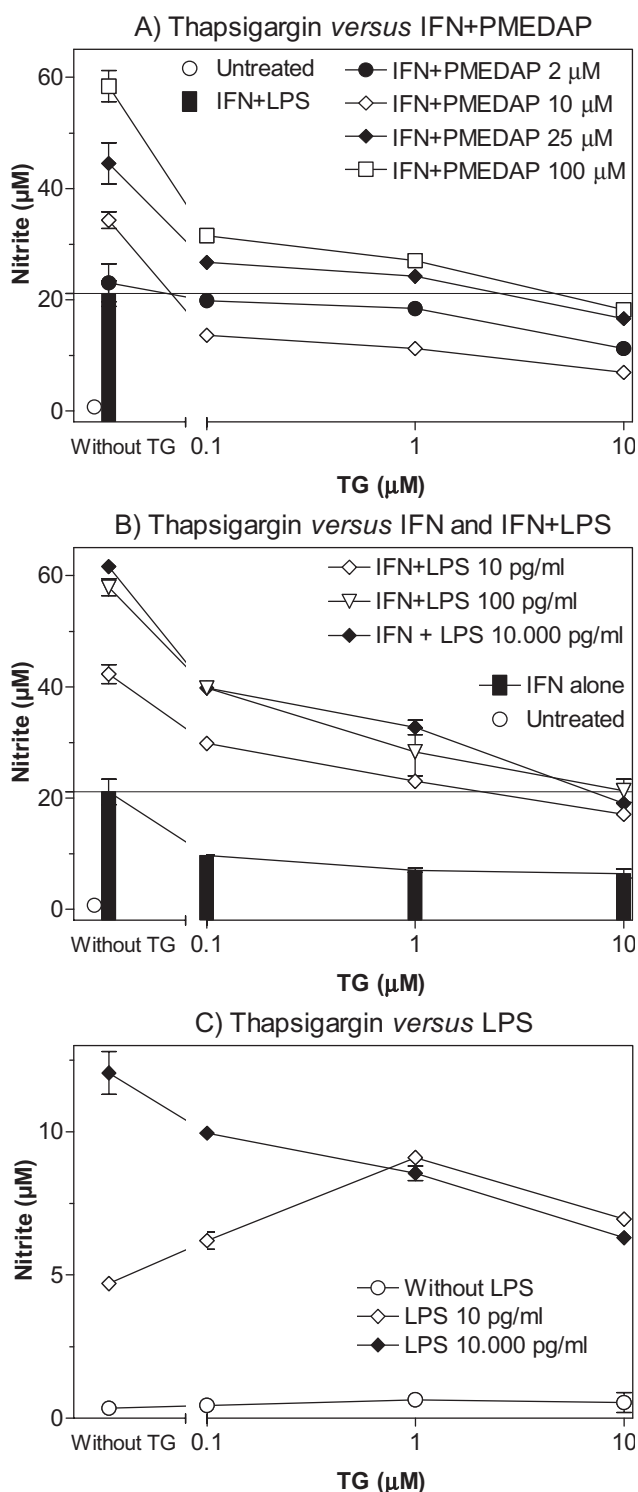
A) Thapsigargin *versus* IFN+PMEDAP

B) Thapsigargin *versus* IFN and IFN+LPS

C) Thapsigargin *versus* LPS

Fig. 2. Interference of thapsigargin (TG) with production of NO by mouse macrophages. NO was activated by IFN-γ, LPS, N^6-isobutyl-PMEDAP (PMEDAP) and their combinations. Thapsigargin was added 30 min before. Supernatant concentration of nitrites was determined after the 24-h culture. Each point is mean ± SEM and data are representative of two identical experiments.

of intracellular calcium plays a role in the LPS-induced expression of TNF-α in macrophage cell line J774.1 (ref.[5]). However, an opposite effect was observed in hepatocytes where TG, in dependence on experimental conditions, did not block or rather inhibited release of TNF-α (ref.[6]). In accordance with the finding of immunostimulatory potential of TG are data showing an enhancement of IL-8 production by human colonic epithelial cells[7], and IL-6 by rat[8], and mouse[9] peritoneal macrophages. In addition, the release of calcium from endoplasmic reticulum stores has been found to activate secretion of IL-1α and IL-1β in murine macrophages[10]. We found that TG had virtually no effect on the immune-stimulated secretion of chemokine MIP-1α/CCL3, although it did activate secretion of the chemokine in immune-nonstimulated macrophages. In sharp contrast, TG profoundly inhibited production of chemokine RANTES/CCL5 as well as antiinflammatory cytokine IL-10 (Fig. 1).

Although secretion of the major NO-upregulatory cytokine TNF-α was enhanced, production of NO was deeply suppressed by TG (Fig. 2) in combination with IFN-γ, IFN-γ/LPS, PMEDAP, IFN-γ/PMEDAP, and with relatively high dose of LPS (10 ng/ml). Notably, as low concentration of TG as 0.1 μM was already effective. On the other hand, production of NO following the low dose of LPS (10 pg/ml) was significantly potentiated by TG. The data are in agreement with findings demonstrating both NO-stimulatory[11] and NO-inhibitory[12] effects of
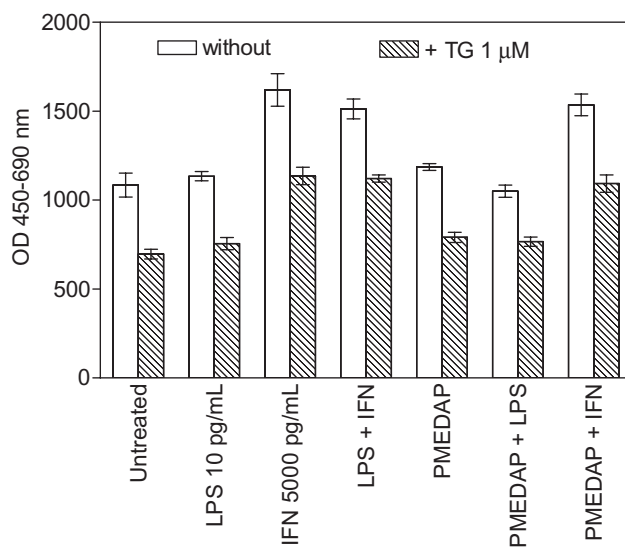

Fig. 3. Effect of thapsigargin on formation of formazan from the tetrazolium salts by mouse macrophages. The WST-1 assay was done after the 20-h culture of cells in the presence of IFN-γ, LPS, and N^6-isobutyl-PMEDAP (PMEDAP; 50 μM) ± thapsigargin (1 μM). Each bar represents mean ± SEM obtained from six culture wells.

TG on iNOS mRNA expression and NO biosynthesis in murine macrophages.

We have found that TG decreases formation of formazan from the tetrazolium salts (Fig. 3), suggesting its toxic influence in terms of decreased number of metabolically active cells in the culture. The suppression was very similar in all experimental settings, reaching statistically highly significant over-all value of 30% ($F_{/1,6/}$ = 250; P < 0.0001). With respect to the fact that not only inhibitory but also stimulatory effects of TG have been observed, the cytotoxicity of TG is unlikely to explain its immunomodulatory interventions.

It can be concluded that TG interferes with cell immune functions. Our original data show that it also interferes with expression of chemokines. The antithetical effects suggest that TG may target multiple underlying signaling pathways. Toxicological aspects of the dual mode of action remain to be firmly established.

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