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Inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPase thapsigargin stimulates production of nitric oxide and secretion of interferon-gamma

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ABSTRACT

Thapsigargin is a sesquiterpene lactone of guaianolide type isolated from the Mediterranean plant *Thapsia garganica* L. It is widely used experimentally as a potent and selective inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) leading to rapid elevation of intracellular calcium $[Ca^{2+}]_i$. Several previous reports have shown that thapsigargin interferes with production of nitric oxide (NO) by mouse peritoneal macrophages and mouse macrophage cell lines. The present data confirm that thapsigargin is a modest inducer of NO in mouse macrophages, production of NO being slightly enhanced by lipopolysaccharide. However, thapsigargin on its own very potently induces NO in macrophages of rats under conditions in vitro. The highest effect was observed after the concentration of 0.25 μM thapsigargin, producing approximately 30 μM accumulation of nitrites in supernatants of cells cultured for 24 h. The aim of our experiments was to investigate immune mechanisms implicated in activation of high-output NO biosynthesis. It has been found that thapsigargin dose-dependently induces secretion of interferon-γ (IFN-γ) in macrophages of both rats and mice, and also in human peripheral blood mononuclear cells. The IFN-γ production was rather low in macrophages of mice while relatively very high levels of IFN-γ were found in cultures of rat macrophages and human peripheral blood mononuclear cells. The concentration of IFN-γ produced by 5 μM thapsigargin within the interval of 24 h exceeded 3 ng/ml in rat macrophages and approached 2 ng/ml in cultures of human peripheral blood mononuclear cells. The effects are mediated by mitogen-activated protein kinases (MAPKs) such as p38 mitogen-activated protein kinase (p38) and extracellular signal-regulated kinases 1/2 (ERK1/2), and by nuclear transcriptional factor NF-κB. In summary, the original findings demonstrate immunostimulatory potential of thapsigargin and warrant more detailed preclinical studies.

1. Introduction

Thapsigargin is a sesquiterpene lactone of guaianolide type, isolated from the Mediterranean plant *Thapsia garganica* L. It is widely used experimentally as a potent and selective inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) leading to rapid elevation of intracellular calcium $[Ca^{2+}]_i$ (Treiman et al., 1998). It inhibits all of the SERCA isozymes with subnanomolar potency (Christensen et al., 1993).

Beside its use as an experimental tool, thapsigargin possesses several pharmacologically interesting properties. Since it is able to kill slowly proliferating and non-proliferating cells, prodrugs of thapsigargin, which are currently undergoing preclinical evaluation, have been developed as novel means for anti-cancer therapy, notably for the treatment of prostate cancer (Denmeade et al., 2003). Furthermore, compounds disrupting the Ca^{2+} homeostasis in endoplasmic reticulum (ER), including thapsigargin, have been shown to possess virustatic activity. Thapsigargin inhibits production of infectious cytomegalovirus (CMV) virions (Isler et al., 2005), replication of hepatitis C virus (HCV) (Nakagawa et al., 2005), herpes simplex virus (HSV) (Cheshenko et al., 2003), and Sendai virus (HVJ) (Ono and Kawakita, 1994). It has been suggested that antiviral effects of thapsigargin may depend on the activation of unfolded protein response which is detrimental to viral infection (Isler et al., 2005; Nakagawa et al., 2005). Anyhow several data can be regarded as a sound argument for the possibility that activation of the immune defence might be attributable to other, so far not revealed effector mechanisms.

One of the major factors inhibiting replication of a number of viruses via several plausible pathways encompassing inhibition of ribonucleotide reductase activity, generation of tricarboxylic acid cycle intermediates, and suppression of an intermediate-early transactivator protein, *Zta*, is nitric oxide (NO) (Karupiah and Harris, 1995; Mannick et al., 1995; Melkova and Esteban, 1995). NO is effective against all poxviridae, herpetoviridae, rhabdoviridae, retroviridae, and parvoviridae, including CMV (He et al., 1995), HCV (Sharara et al., 1997), HSV (Nathan and Hibbs, 1991) and many others (Karupiah and

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Harris, 1997), although not all viruses including e.g. HVJ (Yoshitake et al., 2004) are sensitive to the virucidal activity of NO in vitro.

Thapsigargin has been shown to interfere with nitric oxide (NO) biosynthesis. The effects of thapsigargin on expression of inducible NO synthase (iNOS) mRNA and NO production have frequently been studied in combination with various NO primary activators such as lipopolysaccharide (LPS) (Chen et al., 2005; Jordan et al., 1995; Kiemer and Vollmar, 2001; Korhonen et al., 2001; Park et al., 1996; Park et al., 1995), interferon-γ (IFN-γ) (Raddassi et al., 1994), and interleukin-β (IL-1β) (Geng and Lotz, 1995). The findings are controversial, showing both mild stimulation (Jun et al., 1996; Kmoníčková et al., 2005; Park et al., 1996; Park et al., 1995; Raddassi et al., 1994) and inhibition (Geng and Lotz, 1995; Chen et al., 2005; Jordan et al., 1995; Kiemer and Vollmar, 2001) of NO in mouse peritoneal macrophages or mouse macrophage cell lines. The reasons for the discrepancy are not clear but they seem to depend on the type and intensity of cell stimulation (Kmoníčková et al., 2005; Korhonen et al., 2001).

The aim of the present experiments was to analyze underlying mechanisms determining the interference of thapsigargin with NO biosynthesis. The attention has been focused on possible role of cytokines, key activators of high-output NO production (Bogdan et al., 1994). The findings demonstrate unequivocally that thapsigargin activates secretion of IFN-γ in rat and mouse macrophages as well as in human peripheral blood mononuclear cells.

2. Materials and methods

2.1. Origin of cells and their culture

Female rats of the inbred strain LEWIS, 165–180 g of weight, and female mice of the inbred strain C57BL/6, 8–11 wks old, were purchased from Charles River Deutschland (Sulzfeld, Germany). They were kept in transparent plastic cages and maintained in an Independent Environmental Air Flow Animal Cabinet (ESI Flufrance, Wissous, France). Lighting was set on 06 to 18 h, temperature at 22 °C. Animals, killed by cervical dislocation, were i.p. injected with 8 ml (mice) or 16 ml (rats) of sterile saline. Pooled peritoneal cells collected from 4–8 mice and peritoneal cells from individual rats were washed, resuspended in culture medium, and seeded into 96-well round-bottom microplates (Costar) in final 100-μl volumes, 2×10^6 cells/ml. Adherent cells (macrophages) were isolated by incubating the cells for 2 h at 37 °C, 5% CO_2, and then vigorously shaking the plate and washing the wells three times to remove non-adherent cells.

The sources of human peripheral blood mononuclear cells were buffy coats acquired from healthy donors (provided by the Institute of Hematology and Blood Transfusion, Prague). Peripheral blood mononuclear cells were separated by Ficoll-Paque gradient centrifugation (GE Healthcare Bio-Sciences, AB, Uppsala, Sweden) according to the manufacturer instructions.

The cells were seeded into 96-well round-bottom microplates (Costar, Cambridge, MA) and maintained at 37 °C, 5% CO_2 in humidified Heraeus incubator. The animal macrophages were cultured at final density of 2.0×10^6/ml, the human peripheral blood mononuclear cells at density of 1.0×10^6/ml (100–240 μl/well) in complete RPMI-1640 culture medium. It contained 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 50 μg/ml gentamicin, and 5×10^{-5} M 2-mercaptoethanol (all Sigma-Aldrich, Prague, CR). All protocols were approved by the Institute ethics committee.

2.2. Thapsigargin and other reagents

Thapsigargin was purchased from Sigma. It was prepared as a 25 mM stock solution in DMSO.

The following antagonists of histamine receptors (Sigma) were used: histamine H_1 receptor antagonist pyrilamine maleate, histamine H_2 receptor antagonist cimetidine, histamine H_3 receptor antagonist clobenpropit, and histamine $H_{3/4}$ receptor antagonist thioperamide. The 50 mM stock solutions were prepared in phosphate buffered saline (PBS). Required working concentrations were prepared by diluting the stock solution in complete RPMI-1640 culture medium (described above). The H_3 receptor antagonist clobenpropit was found to be cytocidal at concentrations higher than 10 μM; the others could be used at concentration up to 100 μM since they were devoid of cytotoxicity (data not shown).

The arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (NOHA) (Tenu et al., 1999) was obtained from Bachem (Weil am Rhein, Germany). The 50-mM stock solution was prepared in PBS and added to the macrophage cultures 30 min before thapsigargin. No cytocidal effects of NOHA were found up to the concentration of 250 μM (data not shown).

The following selective inhibitors of mitogen-activated protein kinases (MAPKs), which were obtained from Tocris, Ellisville, MO, were used: a) p38 mitogen-activated protein kinase (p38) inhibitors SB 202190, i.e., 4-[4-(4-fluorophenyl)-5-(4-pyridinyl)-1*H*-imidazol-2-yl] phenol and SB 203580, i.e., 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl) phenyl]-1*H*-imidazol-4-yl]pyridine, b) extracellular signal-regulated kinases 1/2 (ERK1/2) inhibitors PD 98059, i.e., 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one and U0126, i.e., 1,4-diamino-2, 3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene, and c) cJun N-terminal kinase (JNK) inhibitor SP 600125, i.e. anthra[1-9-cd]pyrazol-6(2*H*)-one. The working RPMI-1640 solutions of the inhibitors were prepared from the 10 mM stock solutions in dimethyl sulfoxide (DMSO). The final 10 μM concentrations were not cytotoxic (data not shown).

The inhibitor of transcriptional factor nuclear factor-κB (NF-κB), pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich), was prepared as a 5 mM stock solution in PBS.

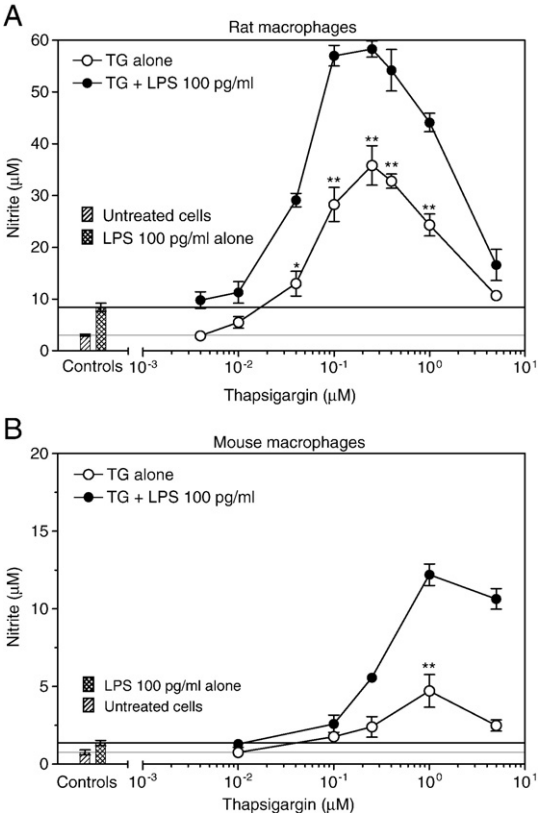

Fig. 1. Effect of thapsigargin (TG) alone or in combination with lipopolysaccharide (LPS; 100 pg/ml) on production of NO by peritoneal macrophages of rats (A) and mice (B). The cells were cultured 24 h in density of 2×10^6/ml. The supernatant nitrite concentration was determined by Griess reagent. Data are means±S.E.M. and represent one of three identical experiments. Statistical analysis was done using one-way ANOVA followed by Dunnett's multiple comparison post-test, * $P<0.05$, ** $P<0.01$.

2.3. Cell viability assay

Viability of cells was determined using a colorimetric assay based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Roche Diagnostics, Mannheim, Germany). The cells were cultured as described above. After the 24-h culture, the WST-1 was added and the cells were kept in the Heraeus incubator at 37 °C for additional 4 h. Optical density at 450–690 nm was evaluated.

2.4. Sterility and lipopolysaccharide (LPS) contamination assay

The samples were sterile filtered using non-pyrogenic 0.22 μm filters (Costar). The chromogenic Limulus Amoebocyte Lysate assay (Kinetic-QCL; Cambrex Bio Science, Walkersville, MD) was used to check for possible contamination with LPS. The final concentrations of LPS in culture wells contained <10 pg/ml of LPS, an amount that is virtually ineffective to activate production of NO and secretion of cytokines (Zídek et al., 2003).

2.5. NO and IFN-γ assay

The cells were cultured 24 h in presence of thapsigargin that was applied either alone or in combination with lipopolysaccharide (LPS; *Escherichia coli* O55:B5, Cambrex Bio Science). The concentration of nitrites in supernatants of cells was taken as a measure of NO production (Marletta et al., 1988). It was detected in individual, cell-free samples (50 μl) incubated 5 min at ambient temperature 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. If indicated, nitrates were reduced to nitrites using nitrate reductase according to standard procedures (Guevara et al., 1998).

Concentration of IFN-γ (pg/ml) in supernatants of cells was determined by enzyme-linked immunoabsorbent assay (ELISA) kit, following the manufacturer instructions (R&D Systems).

2.6. Statistical analysis

Analysis of variance (ANOVA) with subsequent Dunnett's or Bonferroni's multiple comparison tests, analysis of correlations and graphical presentation of data were done using the Prism program (GraphPad Software, San Diego, CA).

3. Results

3.1. Production of NO

Thapsigargin dose-dependently increased the 24-h production of NO by rat peritoneal macrophages (Fig. 1A). The mild effect was observed at low concentration as 0.04 μM, the peak being reached at approximately 0.25-μM concentration of thapsigargin. The response declined afterwards, and was barely detectable at the highest concentration used, i.e. 5 μM. Macrophages from mice (Fig. 1B) were much less responsive to the NO-stimulatory action of thapsigargin. Statistically significant albeit very low production of NO was activated in them with concentration of 1 μM thapsigargin. The NO production induced by thapsigargin alone was statistically, highly and significantly enhanced ($P<0.0001$) in the presence of LPS in both rats and mice (Fig. 1A and B, respectively).

3.2. Secretion of IFN-γ

Upon stimulation with thapsigargin, rat macrophages secreted high amounts of IFN-γ within the interval of 24 h. The IFN-γ production became apparent already after the thapsigargin dose of 0.04 μM, the plateau being reached at approximately 1-μM concen-

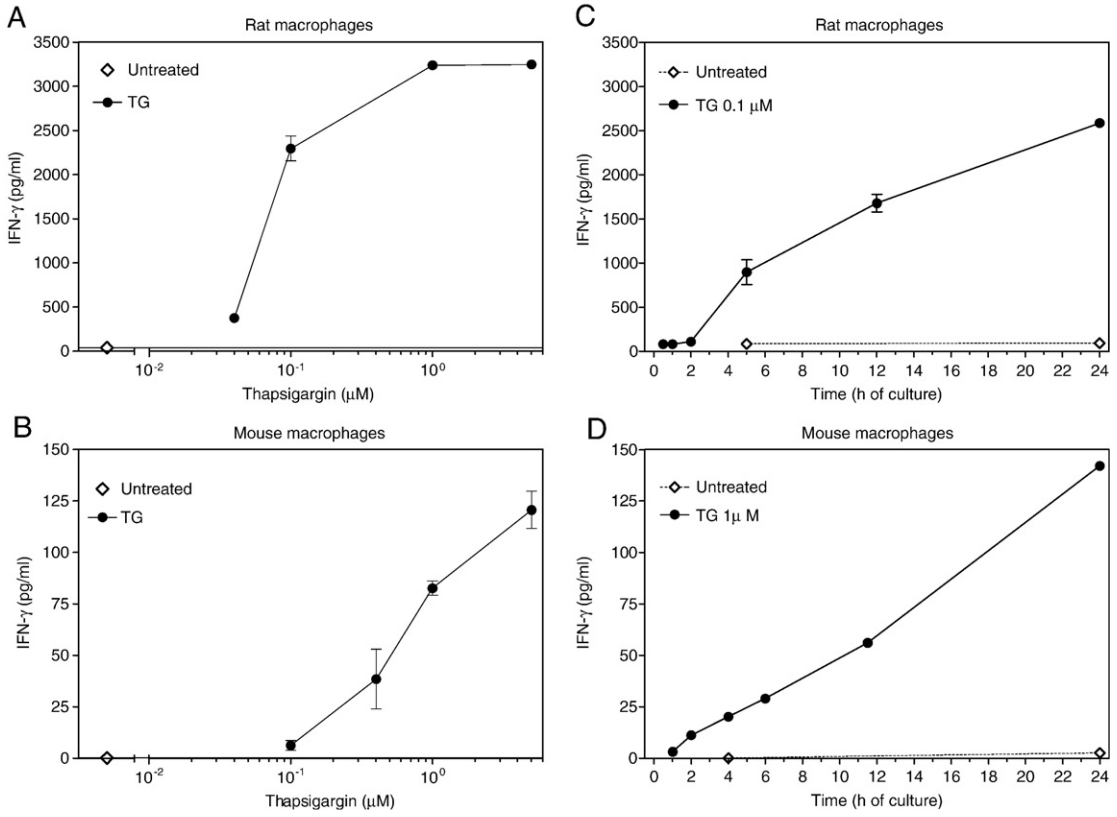

Fig. 2. Secretion of interferon-γ (IFN-γ) by rat (A) and mouse (B) peritoneal macrophages (2×10^6/ml) cultured 24 h in the presence of varying concentrations of thapsigargin (TG). The concentration of IFN-γ depends on the time interval successive to TG administration (C and D for rats and mice, respectively). IFN-γ in cell supernatants was determined by ELISA. Each point is a mean±S.E.M. and data represent one of two identical experiments.

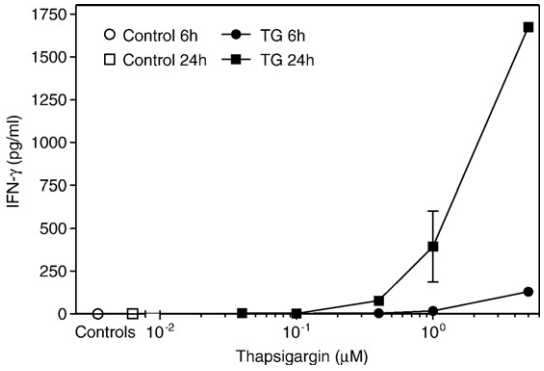

Fig. 3. Secretion of IFN-γ by human peripheral blood mononuclear cells (3×10^6/ml) in dependence on concentration of thapsigargin (TG). Levels of IFN-γ were evaluated after 6 or 24 h of cell culture. IFN-γ in supernatants was determined by ELISA. Each point is a mean ± S.E.M. obtained from two donors of cells, cultured in duplicate.

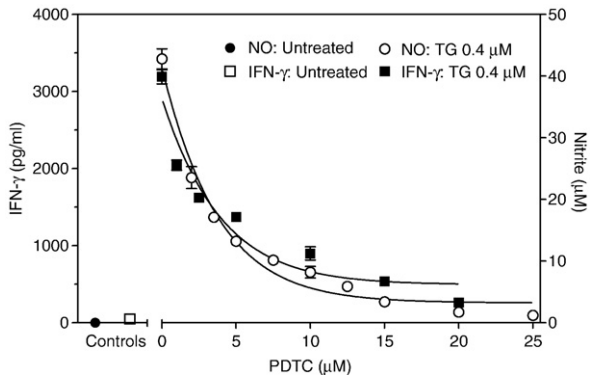

Fig. 5. Inhibitory effects of the inhibitor of NF-kB, pyrrolidine dithiocarbamate (PDTC), on production of nitric oxide and IFN-γ. Rat peritoneal macrophages (2×10^6/ml) were cultured 24 h in the presence of 0.4 μM thapsigargin (TG). PDTC was added 15 min before TG. The points represent means ± S.E.M.

tration of thapsigargin (Fig. 2A). The onset of augmented IFN-γ production was within the period of 2–4 h following thapsigargin administration (Fig. 2C). Mice macrophages were activated to secrete IFN-γ too, but the extent of production was much lower than in rats (Fig. 2B—dose dependence; Fig. 2D—time dependence).

Thapsigargin induced considerable amounts of IFN-γ also in human peripheral blood mononuclear cells (Fig. 3). The increase was much more pronounced in supernatants of cells cultured for 24 h than for 6 h, the dose of 5 μM thapsigargin being the most effective.

3.3. Involvement of MAPKs

The specific inhibitors of MAPKs p38 (SB 202190, SB 203580) and ERK1/2 (U0126, PD 98059) significantly ($P<0.05$–0.01) suppressed secretion of IFN-γ in both rat and mouse peritoneal macrophages stimulated in vitro with thapsigargin (Fig. 4). The effects of p38 MAPK inhibitors were more prominent than the effects of ERK1/2 ones. The JNK MAPK inhibitor (SP 600125) did not change the thapsigargin-induced production of IFN-γ. DMSO which was used as a solvent for the MAPK inhibitors, and which was applied in an amount corresponding to its final presence in MAPKs solutions, did not interfere with production of thapsigargin-induced IFN-γ. The 10-μM concentrations of the MAPK inhibitors did not show any cytotoxicity as proved by the cell viability WST-1 assay (data not shown).

3.4. Involvement of NF-κB

The inhibitor of NF-κB activation, PDTC, inhibited production of both IFN-γ and NO (Fig. 5) induced in rat macrophages by thapsigargin (0.4 μM). Concentrations of PDTC necessary for 50% response inhibition (IC_{50}s) were very similar in both cases: 3.14 μM (95% limits of confidence: 1.18–8.32) and 3.98 μM (95% limits of confidence: 2.47–6.fs42) for IFN-γ and NO, respectively.

3.5. Cell viability

The cells were exposed to thapsigargin for 24 h. Up to the concentration of 1 μM, thapsigargin had no effect on viability of rat and mouse macrophages (Fig. 6) as assayed by the cleavage of tetrazolium salt WST-1 by mitochondrial dehydrogenases. The 5-μM thapsigargin suppressed the viability of rat and mouse macrophages by 28% ($P<0.01$) and 25% ($P<0.05$), respectively. The decrease was more pronounced (by 37%, $P<0.01$) in mouse macrophages treated with 10-μM thapsigargin.

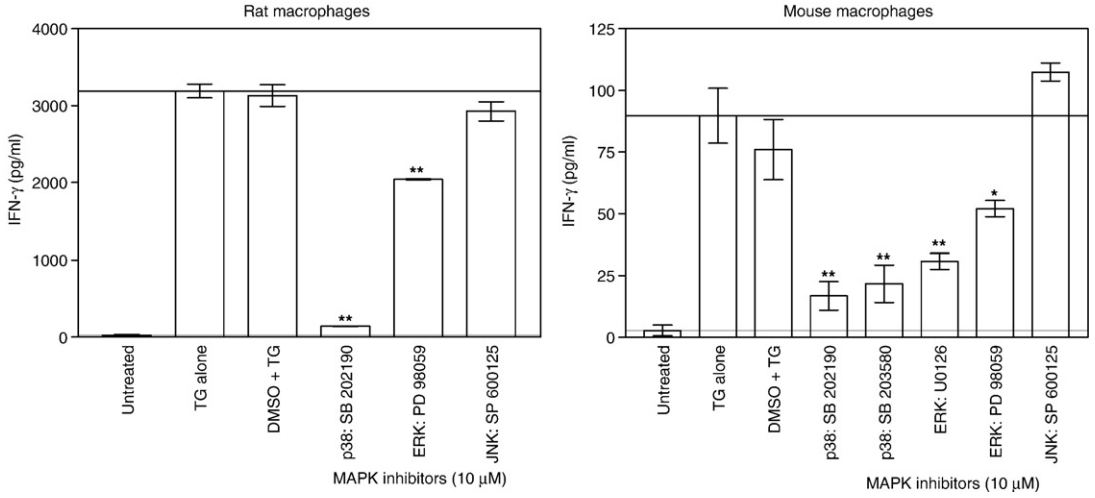

Fig. 4. Inhibitory effects of p38, ERK1/2 and JNK MAPKs inhibitors on secretion of IFN-γ by rat and mouse peritoneal macrophages (2×10^6/ml) cultured 24 h in the presence of thapsigargin (TG). The dose of TG was 0.4 μM and 1.0 μM in rat and mouse cell cultures, respectively. The inhibitors were applied at 10-μM concentration 15 min before TG. Levels of IFN-γ were evaluated by ELISA. The bars are means ± S.E.M., representing one of two identical experiments. Statistical analysis was done using one-way ANOVA followed by Dunnett's multiple comparison post-test, * $P<0.05$, ** $P<0.01$.

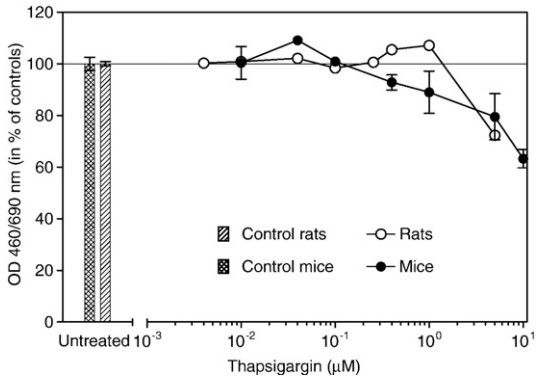

Fig. 6. Effects of thapsigargin (TG) on viability of rat and mouse peritoneal macrophages using a colorimetric assay based on cleavage of the tetrazolium salt WST-1. The cells were cultured in the density of $1\times1\ 0^6$/ml for 24 h. The WST-1 was added for the next 4 h. Optical density was evaluated in percent of control values. Each point is mean ± S.E.M. and the data are typical for three other similar experiments.

3.6. Role of arginase

The inhibitor of arginase N^{ω}-hydroxy-nor-L-arginine (NOHA), applied in a broad range of concentrations (10–100 μM), was unable to alter production of NO (Fig. 7) that was evaluated at the interval of 24 h following administration of thapsigargin. Rat peritoneal macrophages cultured at the density of 2×10^6/ml were used for this experiment. By itself, NOHA did not influence the spontaneous production of NO.

3.7. Role of histamine

None of the histamine receptor antagonists employed, i.e., pyrilamine (histamine H_1 receptor antagonist), cimetidine (histamine H_2 receptor antagonist), clobenpropit (histamine H_3 receptor antagonist), and thioperamide (histamine $H_{3/4}$ receptor antagonist) significantly influenced biosynthesis of NO activated by thapsigargin (1 μM) in rat and mouse peritoneal macrophages. The antagonists were applied 10 min prior to thapsigargin. The antagonists did not interfere with NO production on their own, as exemplified by unchanged NO production by rat macrophages (Fig. 8). Similarly, the thapsigargin-stimulated IFN-γ production in rat macrophages remained uninfluenced by histamine receptors antagonists within the 24 h of culture (Fig. 9).

4. Discussion

One of the factors controlling activation of all types of NO synthase (NOS) isoforms resulting in augmented NO production is concentration of intracellular free calcium $[Ca^{2+}]_i$. For example, glutamate binding to *N*-methyl-D-aspartate (NMDA) receptors increases levels of $[Ca^{2+}]_i$, which in turn activates neuronal NOS (nNOS) via calmodulin for the mediation of rapid events such as neurotransmission (Dawson and Snyder, 1994). Activation of endothelial NOS (eNOS) in endothelial cells is associated with enhanced NO production and depends on elevation of $[Ca^{2+}]_i$ (Zheng et al., 2006). The traditionally considered independency of inducible NOS (iNOS) activity on calcium has been observed in a number of experiments, e.g. Geng and Lotz (1995). Yet, its activity can be positively regulated by $[Ca^{2+}]_i$ under various experimental situations, such as simultaneous administration of LPS and UTP to the mouse macrophage cell line J774 (Chen et al., 1998) and culture of macrophages with estradiol (Azenabor et al., 2004). Human hepatocyte iNOS looses activity after Ca^{2+} chelation and exposure to calmodulin antagonist (Geller et al., 1993).

The increase of intracellular Ca^{2+} evoked by thapsigargin has been suggested to provide a priming signal leading ultimately to enhanced production of NO (Jun et al., 1996). Our present data confirm the previous findings showing that thapsigargin is a weak activator of NO biosynthesis in mouse macrophages (Jun et al., 1996; Kmoníčková et al., 2005; Park et al., 1996; Park et al., 1995; Raddassi et al., 1994). We demonstrate here for the first time, that the NO-stimulatory action of thapsigargin is much more pronounced in macrophages of rats. While the highest level of NO production by mouse macrophages does not exceed 5 μM following the dose of 1-μM thapsigargin, the peak NO concentration is about 7-fold higher following the concentration of 0.25-μM thapsigargin in macrophages of rats. Another characteristic pattern of the thapsigargin-triggered NO formation is its enhancement by concomitantly applied LPS. This is in agreement with findings of Park et al. (1996, 1995) showing that thapsigargin alone has negligible effect on NO synthesis in murine peritoneal macrophages, whereas both iNOS mRNA and NO synthesis are dose-dependently increased by thapsigargin in combination with LPS. Interestingly, also LPS induces transient increase of intracellular free calcium $[Ca^{2+}]_i$. It remains elevated for 30 min after LPS treatment of rat peritoneal cells (Hotchkiss et al., 1997). The $[Ca^{2+}]_i$ increase after stimulation of rat macrophages with LPS has been suggested to be due to the phosphorylation of phospholipase C (PLCγ1/2) which is mediated by protein-tyrosine kinase. Activation of several protein kinases C (PKC) follows and leads to the regulation of serine phosphorylation in MEKK1 by PKCβ. These steps result in activation of NF-κB and ultimately in promotion of iNOS activity (Zhou et al., 2006).

A number of cytokines can influence the high-output production of NO. However, the crucial function is possessed by IFN-γ that triggers NO formation on its own in many cell types and under many experimental conditions (Stuehr and Marletta, 1987). It is recognized that IFN-γ production is also central to the LPS-mediated induction of iNOS mRNA, and that the NO-upregulatory effect of LPS is mainly due to the synergistic cooperation with IFN-γ (ter Steege et al., 1998). The synergism may result from several reasons. Although LPS activates the NF-κB family of transcription factors (Muller et al., 1993) and the signal transduction of IFN-γ is mediated by JAK-STAT pathway (Darnell, 1998), both of them are activators of interferon regulatory factor-1 (IRF-1), a key inducer of iNOS expression (Kamijo et al., 1994). Furthermore, LPS may stabilize iNOS mRNA: while the stability is about 1–1.5 h after IFN-γ activation alone, it is increased to 4–6 h in the presence of LPS (Weisz et al., 1994).

These facts made us hypothesize that the enhancing effects of thapsigargin on NO production might result from its ability to activate secretion of IFN-γ. Indeed, the results show unequivocally that thapsigargin does induce IFN-γ in all mouse and rat peritoneal macrophages, and in human peripheral blood mononuclear cells. While constitutive IFN-γ amounts are negligible irrespective of the

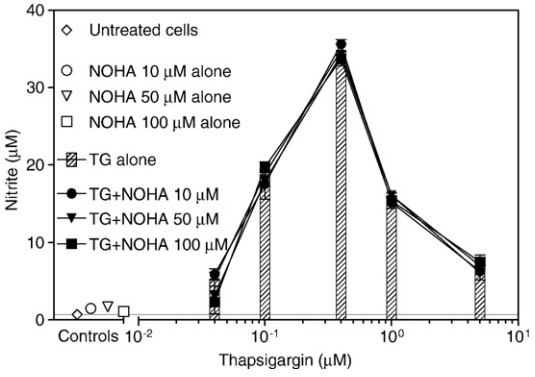

Fig. 7. The 24-h production of NO by rat peritoneal macrophages (2×10^6/ml) stimulated with increasing concentrations of thasigargin (TG) and cultured without (bars) or in concomitant presence (lines) of the inhibitor of arginase N^{ω}-hydroxy-nor-L-arginine (NOHA). The NOHA was applied 30 min before TG. Each point is a mean ± S.E.M. of triplicate assay.

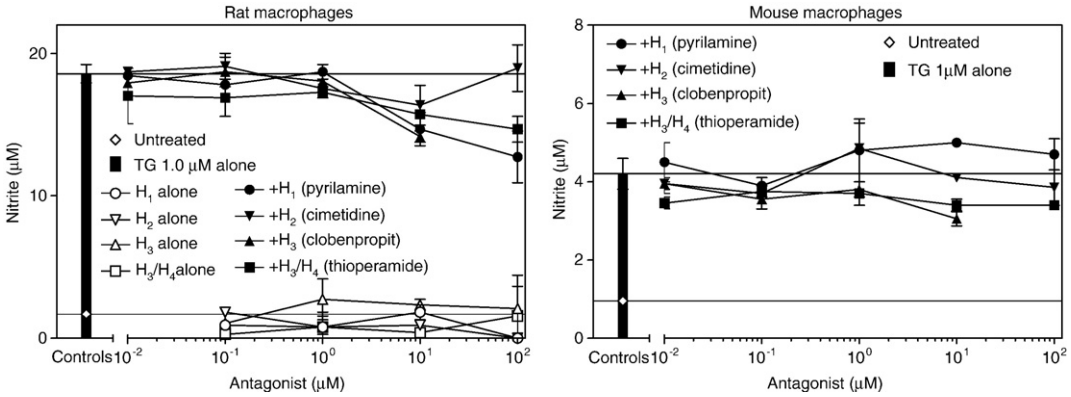

Fig. 8. Effects of histamine receptor antagonists on production of NO induced in rat and mouse peritoneal macrophages (2×10^6/ml) by thapsigargin (TG; 1.0 μM). The antagonists were applied 10 min prior to TG. No significant influence of the antagonists was found (evaluated by analysis of variance and subsequent Bonferroni's multiple comparison test). The data are means ± S.E.M. and are representative of two identical experiments.

origin of cells, the increase is apparent within the interval of 2–6 h in rodent macrophages. The 24-h interval is optimal for accumulation of IFN-γ in cultures of human peripheral blood mononuclear cells. The highest levels of IFN-γ were produced by macrophages of rats and by human peripheral blood mononuclear cells, whereas macrophages of mice showed relatively modest production of IFN-γ within the 24-h interval of culture.

Production of both IFN-γ and NO was inhibited by inhibitors of MAPKs p38 and ERK1/2 and inhibitor of NF-κB. It may be suggested therefore that thapsigargin is a potent activator of these underlying signaling pathways.

The extent of IFN-γ secretion corresponds to the thapsigargin-triggered NO response in mice (low responders) and rats (high responders). We did not find any NO production in human cells (data not shown). The absence of NO biosynthesis could be anticipated in this case, because despite of greatly enhanced systemic NO production in humans under various patho-physiological conditions (Hibbs et al., 1992; Ochoa et al., 1991; Schön et al., 1999), human cells are notoriously known to be refractory to common in vitro NO-enhancing stimuli (Zhang et al., 1996).

Secretion of IFN-γ behaved in a strict dose-dependent manner. In contrast, production of NO exhibited a "bell-shaped" curve, confirming thus previously described findings (Jun et al., 1996). Nitrite concentration gradually increased up to the maximum response

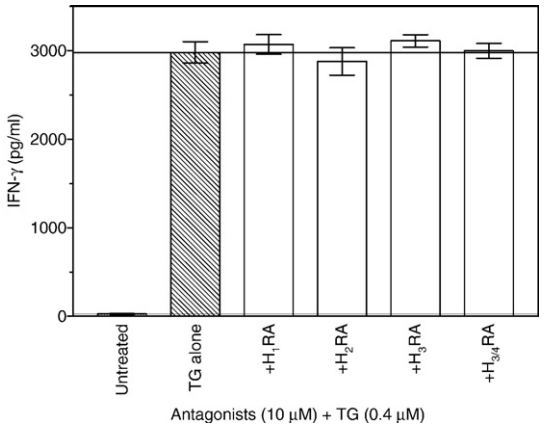

Fig. 9. Histamine receptor antagonists (HRA; 10 μM) remained without any significant effect on secretion of IFN-γ by rat peritoneal macrophages (2×10^6/ml) cultured for 24 h in the presence of thapsigargin (TG, 0.4 μM). The antagonists were applied 10 min prior to TG. The following histamine receptor antagonists were used: pyrilamine (H_1RA), cimetidine (H_2RA), clobenpropit (H_3RA), and thioperamide ($H_{3/4}$RA). IFN-γ was determined using ELISA in supernatants of cells after the 24-h culture. The bars are means ± S.E.M.

reached with approximately 0.25 μM and 1.0 μM concentration of thapsigargin in macrophages of rats and mice, respectively. However, the NO production dropped afterwards towards the highest, i.e., 5-μM dose of thapsigargin. In an attempt to find out plausible explanation for this phenomenon, we analyzed possible involvement of several factors that a) are related to other biological activities of thapsigargin, i.e., its cytotoxicity and effects on histamine production, or b) might be relevant to NO production in general, i.e., a role of arginase.

Concerning cytotoxicity, no cytocidal effects were observed up to the 1-μM in vitro concentration of thapsigargin in cultures of both rat and mouse macrophages. Viability of the cells was reduced by 28% and 21%, respectively, with the 5-μM concentration of thapsigargin. The 10-μM thapsigargin concentration was more toxic, producing approximately 37% decrease of viability of mouse macrophages. The findings are in good line with literature data showing that cell viability in human liver-derived cell line Huh7 was unchanged within 24-h culture after thapsigargin up to 5 μM concentration, and it was only slightly decreased at concentration of 10 μM of thapsigargin (Xie et al., 2002).

Thapsigargin has been reported to induce the expression of L-histidine decarboxylase (HDC) which is responsible for the synthesis of histamine from L-histidine (Shiraishi et al., 2000). Concentration-dependent production and release of histamine after thapsigargin treatment have been observed in many cell types such as a murine macrophage-like cell line RAW 264 (Shiraishi et al., 2000), rat mast cells (Ohuchi et al., 1989), human lung mast cells (Yang et al., 2006) and human basophil leukocytes (Ali et al., 1985). Also resident macrophages which express HDC are producers of histamine (Zwadlo-Klarwasser et al., 1998). For example, mouse bone marrow-derived macrophages (Takamatsu et al., 1996) and mouse peritoneal macrophages expressing on the surface both histamine H_1 and H_2 receptors (Okamoto and Nakano, 1990) produce histamine through the LPS-induced activation of HDC. Histamine is one of the factors that may participate on regulation of NO biosynthesis, though the data on its influence are controversial. It has been found to inhibit NO production in rat alveolar macrophages (Miles et al., 1998), LPS-pretreated rat mast cells and in bradykinin-pretreated guinea pig hearts (Mannaioni et al., 1997). On the other hand, histamine, and histamine H_2 and H_3 receptor agonists were found to stimulate NO production in LPS-activated alveolar macrophages from humans and rats (Sirois et al., 2000) and in rat mast cells (Carlos et al., 2006). The decisive role is obviously played by histamine H_2 and H_3 receptors, not by histamine H_1 receptors (Sirois et al., 2000). Under the conditions of the present experiments, histamine obviously plays no role in the thapsigargin-induced production of NO. Neither inhibition nor enhancement of NO was observed in macrophages of both rats and mice treated simultaneously with varying concentrations of

thapsigargin plus antagonists of histamine H_1, H_2, H_3, and $H_{3/4}$ receptors. The extent and typical "bell-shaped" profile of NO production in dependence on thapsigargin concentration remained uninfluenced by these histamine receptor antagonists.

The enzyme arginase competes with NOS for the substrate L-arginine, converting it to L-ornithine and urea and limits thus its availability for NO synthesis (Hey et al., 1997). Though arginase appears to occur constitutively, its activity can be altered by various factors. LPS stimulates both iNOS and arginase activity. However there are differences in rats and mice: arginase induced in mouse macrophage RAW 264.7 cell line is the arginase type II (Wang et al., 1995), whereas arginase type I (i.e. liver arginase) is induced in rat peritoneal macrophages. Interestingly, induction of arginase mRNA in rat macrophages by LPS is slower than the induction of iNOS mRNA (Sonoki et al., 1997). Various in vivo inflammatory stimuli, such as treatment of mice and rats with thioglycolate, carrageenan, casein, Bacillus Calmette-Guérin vaccine and Newcastle Disease Virus activated both iNOS and arginase activities in mice, but only iNOS but not arginase activity was changed in rats (Hrabák et al., 2006). Arginase can also be stimulated by cytokine down-regulators of NOS activity such as transforming growth factor-β (TGF-β) (Boutard et al., 1995), IL-4, IL-10, IL-13 (Modolell et al., 1996), and IL-3 (Schneider and Dy, 1985). IFN-γ does not induce arginase (Modolell et al., 1995) but it rather inhibits it (Wang et al., 1995). In our experimental design, inhibition of arginase activity using a specific inhibitor N^{ω}-hydroxy-nor-L-arginine (NOHA) had no effect on production of NO within the broad range of thapsigargin concentrations.

Therefore, the "bell-shaped" character of NO production in dependence on thapsigargin concentration as well as the differences between rat *versus* mouse macrophages in their NO responsiveness cannot be satisfactorily explained by factors such as changes in cell viability and histamine involvement. Neither the arginase activity has proved to be responsible for these phenomena. Since thapsigargin is a highly lipophilic molecule (Treiman et al., 1998), its penetration across the cell membrane is unlikely to influence the distinct patterns of biological effects of thapsigargin either, especially in mice *versus* rats. It has been found recently that along with the existence of several families of Ca^{2+} pumps, the so-called non-muscle SERCA3 represents a heterogeneous sub-family of proteins consisting of many isoforms, the genetic control and function of which may differ (Bobe et al., 2005; Martin et al., 2002). It cannot be excluded that species-specific differences in expression of these isoforms and possibly different affinity of thapsigargin to them might be a key to the better understanding of biological effectiveness of thapsigargin in various species.

In summary, the original data show unequivocally that thapsigargin may be regarded as a novel immunostimulatory agent. It is a potent activator of IFN-γ secretion in both animal and human cell systems. Since endogenous IFN-γ has been implicated in control of growth of an impressive range of obligate and facultative intracellular organisms (Kaufmann, 1993), the results presented here suggest a perspective for further preclinical evaluation of this agent.

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