2,4,6-Trimethyl-*N*-[3-(trifluoromethyl)phenyl]benzenesulfonamide increases calcium influx in lipopolisaccharide-pre-treated arteries

ELŻBIETA GRZEŚK[1], KATARZYNA SZADUJKIS-SZADURSKA[2], ELŻBIETA BLOCH-BOGUSŁAWSKA[3], MICHAŁ WICIŃSKI[2], BARTOSZ MALINOWSKI[2], SYLWIA KOŁTAN[1], BARBARA TEJZA[1], MACIEJ PUJANEK[2] and GRZEGORZ GRZEŚK[2]

Departments of [1]Pediatrics, Hematology and Oncology, [2]Pharmacology and Therapeutics and [3]Forensic Medicine, Collegium Medicum, Nicolaus Copernicus University, PL-85-094 Bydgoszcz, Poland

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Abstract. It has been demonstrated that 2,4,6-trimethyl-*N*-[3-(trifluoromethyl)phenyl]benzenesulfonamide (m-3M3FBS) activates phospholipase C (PLC) and stimulates apoptosis in smooth muscle cells, which may increase vascular reactivity. The primary aim of the present study was to evaluate the physiological effects of the direct stimulation of PLC by m-3M3FBS on vascular smooth muscle reactivity in arteries pre-treated with lipopolysaccharides (LPS) as a model of septic shock. Experiments were performed on isolated and perfused tail arteries of Wistar rats. The contraction force in the model was measured by assessing increases in perfusion pressure at a constant flow. Parameters describing the concentration-response curves (CRCs) obtained for phenylephrine and arginine-vasopressin in the presence of LPS confirmed a decrease in vessels reactivity. In comparison with the controls, m-3M3FBS treatment caused a significant increase in LPS-untreated as well as pre-treated arteries. Furthermore, in the presence of m-3M3FBS, calcium influx from intra- as well as extracellular calcium stores was significantly higher for LPS-untreated and pre-treated arteries. The results of the present study suggested that m-3M3FBS significantly increased the reactivity of vascular smooth muscle cells pre-treated with LPS by increasing the calcium influx from intra- and extracellular calcium stores. Further studies investigating this mechanism are required to evaluate whether this pathway may be a potential therapeutic strategy to treat sepsis.

Correspondence to: Dr Elżbieta Grześk, Department of Pediatrics, Hematology and Oncology, Collegium Medicum, Nicolaus Copernicus University, Ulica Skłodowskiej-Curie 9, PL-85-094 Bydgoszcz, Poland
E-mail: ellag@cm.umk.pl

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Introduction

Nitric oxide synthase (NOS) is an enzyme that catalyzes a reaction which generates nitric oxide in two different stages. The first stage is the oxidation of L-arginine to N-omega-hydroxy-L-arginine, which is degraded to L-citrulline in the second stage by NOS and oxygen, and accompanied by the release of nitric oxide from endothelial cells. There are three main types of nitric oxide synthase: NOS-1, -2 and -3. NOS-2 is localized mainly in macrophages, striated heart muscle, liver, vascular smooth muscle or vascular endothelium, and is activated as a response to infection, inflammation or sepsis following the release of pro-inflammatory cytokines, including interleukin (IL)-1, interferon (IFN)-γ or tumor necrosis factor (TNF)-α. The activated enzyme is active for a few h and synthesizes large quantities of nitric oxide (1). Nitric oxide produced by NOS-3 acts predominantly as a regulator of muscle tension in the local regulation of vascular tone. It is also a factor inhibiting the adhesion and aggregation of platelets, as well as angiogenesis. The role of NOS-3 as part of the initiation of NOS-2 activation in the presence of lipopolysaccharides (LPS) has been investigated over the past decade. The first study suggesting that NOS-3 has a role in the generation of NO-associated hyporeactivity during early sepsis was published in 2001 (2,3). A study on isolated animal tissue treated with short acting LPS (~5 h) showed a statistically significant inhibition of NOS-2 expression following blockade of NOS-3 activity, suggesting that, due to its feedback regulation of LPS-induced NOS-2, nitric oxide synthesized by NOS-3 is a mediator of inflammation in sepsis (4). These results were in accordance with those of another study, which indicated that the absence of NOS-3 inhibits the full expression of NOS-2 in the presence of LPS, and suggested that in the pathogenesis of sepsis, NOS-3 is initially activated and the released nitric oxide appears to stimulate an increase in NOS-2 expression (5).

Phospholipase C (PLC) is the key enzyme activated following stimulation of numerous G-protein-coupled receptors. PLC induces the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate into intracellular diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (6), and modulation of PLC reactivity may alter the cellular response to receptor stimulation. It is possible to directly activate PLC with 2,4,6-trim ethyl-*N*-[3-(trifluoromethyl)phenyl]benzenesulfonamide

(m-3M3FBS) and to thus increase the calcium ion concentration in the cytoplasm (6,7); however, the selectivity of this action remains contested (8-10). Chen *et al* (11) detected increased calcium influx and apoptosis in 3M3FBS pretreated SCM1 human gastric cancer cells and Liu *et al* (12) observed a similar effect in m-3M3FBS pretreated HA59T human hepatoma cells.

m-3M3FBS is responsible for the increase in calcium influx from intra- and extracellular calcium stores that activate G-protein-coupled receptors, including α_1-adrenoceptors and vasopressin receptors, thus stimulating vascular smooth muscle cells. In a previous study by our group, the same increase was found during extra-receptor stimulation, such as during direct stimulation of G-protein with mastoparan-7; however, the presence of m-3M3FBS did not modify the arterial muscle reactivity following direct stimulation of L-type calcium channels with the calcium agonist Bay K8644 (13).

The modulatory effect of m-3M3FBS on smooth muscle reactivity in LPS-pre-treated tissue was first described by an author of the present study in their habilitation thesis (14) and the protective effect of m-3M3FBS was confirmed by Kim *et al* (15).

To the best of our knowledge, no previous study has directly analyzed the effectiveness of stimulating PLC with m-3M3FBS in hyporeactive vascular smooth muscles. Therefore, the aim of the current study was to analyze the constrictive effects of m-3M3FBS on arteries pre-treated with LPS as a model of septic shock in order to evaluate its potential therapeutic application.

Materials and methods

Animal model. Experiments were performed on isolated and then perfused arteries. A total of 16 male Wistar rats (age, ~2 months; Hodowla Zwierząt Laboratoryjnych, Brwinów, Poland) weighing 250-350 g were housed at 20-21˚C and a humidity of 50-60% for a maximum of one day under a 12-h light/dark cycle with *ad libitum* access to water and food. Animals were anesthetized by intraperitoneal injection of 120 mg/kg urethane (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and then sacrificed by cervical dislocation. The protocol of the current study was approved by the Local Ethics Committee for Experiments on Animals (University of Technology and Life Sciences, Bydgoszcz, Poland) and all experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the US National Institute of Health (NIH) from 1985.

Drugs and solutions. Experiments were performed to determine the role of intracellular and extracellular calcium ions in contraction induced by phenylephrine (PHE) and arginine-vasopressin (AVP) under control conditions and in LPS-pre-treated arteries using two types of Krebs fluid: i) Free physiological salt solution (FPSS)-Ca^{2+}-free EGTA-Krebs with the following composition: NaCl (71.8 mM/l), KCl (4.7 mM/l), $MgSO_4$ (2.4 mM/l), $NaHCO_3$ (28.4 mM/l), KH_2PO_4 (1.2 mM/l) and glucose (11.1 mM/l) with added EGTA (30 μM/l; all Sigma-Aldrich; Merck Millipore); and ii) PSS - FPSS with added $CaCl_2$ (1.7 mM/l; Sigma-Aldrich; merck Millipore), which was applied after the intracellular pool of calcium ions was exhausted.

Study design and conduction. Segments of rat-tail arteries (length, 2.5-3.0 cm) were dissected from surrounding tissues. The proximal segment of the artery was cannulated and connected to the perfusion apparatus in a 20-ml isolated organ bath (TSZ-04; Experimetria Ltd., Budapest, Hungary) filled with oxygenated Krebs solution at 37˚C. In the initial section of the experiment, perfusion fluid (FPSS) flow was gradually increased to 1 ml/min. The changes in continuously measured perfusion pressure in the experimental system were an exponent of arterial smooth muscle contractility. Perfusion pressure was measured on BPR-01 and BPR-02 transducers (Experimetria Ltd.) connected to a digital recorder (Graphtec midi Logger GL820; Graphtec Corp., Yokohama, Japan). The peristaltic pump, which was used to induce arterial perfusion, was purchased from ZALIMP (Warszawa, Poland) (16,17). All reagents were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany).

Data analysis and statistical procedures. The classical pharmacometric van Rossum method was used to calculate concentration-response curves (CRCs) (2,3). The maximal effect (E_{max}) of tissue stimulation was calculated as a percentage of the maximal response for the respective agonist. The half-maximal effective dose (ED_{50}) was calculated using previously described pharmacological methods with pD_2 as the negative logarithm of the $ED_{50,}$ according to the van Rossum method (2,3,16). The number of CRC and E_{max} were used in all calculations estimating statistical significance.

Values are expressed as the mean ± standard deviation. The Shapiro-Wilk test was used to determine normal distribution of the investigated variables. Statistical analysis was performed using the Newman-Keuls and analysis of variance test for multiple comparisons of means, using the R statistical software package (version 3.2.2; R Foundation for Statistical Computing, Vienna, Austria). $P<0.05$ was considered to indicate a statistically significant difference.

Results

m-3M3FBS reduces LPS-induced relaxation of AVP- or PHE-stimulated arterial vessels. The CRCs obtained for PHE and AVP were sigmoidal (data not shown). CRCs in the presence of m-3M3FBS were shifted to the left with an increase in maximal responses. For all data-points with a relative effect of ≥20%, the observed leftward shift was statistically significant ($P<0.05$). Calculated EC_{50} values in m-3M3FBS-treated groups were significantly lower than those in the control groups. The opposite effect was seen after addition of LPS with CRCs shifted to the right with a reduction in maximal responses and increase in calculated EC_{50} values ($P<0.05$). In CRCs treated with LPS and m-3M3FBS, a significant rightward shift compared with the CRCs of the control groups was observed; however, compared with the CRCs obtained in the presence of LPS alone, a significant leftward shift was observed ($P<0.05$; Fig. 1). Calculated parameters describing CRCs such as E_{max}, EC_{50} and pD_2 values are presented in Table I.

m-3M3FBS reduces LPS-induced decreases in calcium influx from the intra- and extracellular space in rat arteries. The effect of m-3M3FBS (10^{-5} M/l) on the maximal perfusion

Table I. Impact of phospholipase activator m-3M3FBS on EC_{50}, maximal response and relative potency for AVP and PHE with or without LPS.

Treatment	n	$\%E_{max}$	EC_{50}, M/l	pD_2	RP
AVP	25	100	$1.83\pm0.61x10^{-8}$	7.74±0.14	1.000
AVP + m-3M3FBS	22	119±12	$1.41\pm0.44x10^{-8\,b}$	7.85±0.12	1.298
AVP + LPS	22	75±9	$2.00\pm0.7x10^{-7\,c}$	6.70±0.12	0.092
AVP + LPS + m-3M3FBS	20	87±12	$1.55\pm0.52x10^{-7\,c,d}$	6.81±0.14	0.118
PHE	34	100	$7.54\pm0.95x10^{-8}$	7.12±0.07	1.000
PHE + m-3M3FBS	18	116±12	$6.43\pm2.1x10^{-8\,a}$	7.19±0.18	1.173
PHE + LPS	18	77±9	$6.31\pm1.91x10^{-7\,c}$	6.20±0.17	0.119
PHE + LPS + m-3M3FBS	18	82±10	$4.97\pm1.72x10^{-7\,c,e}$	6.30±0.15	0.152

Comparison with control ($^aP<0.05$, $^bP<0.01$, $^cP<0.0001$); comparison with AVP+LPS ($^dP<0.01$); comparison with PHE+LPS ($^eP<0.05$). Values are expressed as the mean ± standard deviation. n, number of concentration-response curves used for calculations; $\%E_{max}$, calculated as a percentage of maximal response for controls; RP, relative potency, calculated as EC_{50} for controls/EC_{50}; EC_{50}, half maximal effect concentration; E_{max}, maximal tissue response; pD_2, negative logarithm of the EC_{50}; m-3M3FBS, 2,4,6-trimethyl-*N*-[3-(trifluoromethyl)phenyl]benzenesulfonamide; AVP, arginine-vasopressin; PHE, phenylephreine; LPS, lipopolysaccharides.

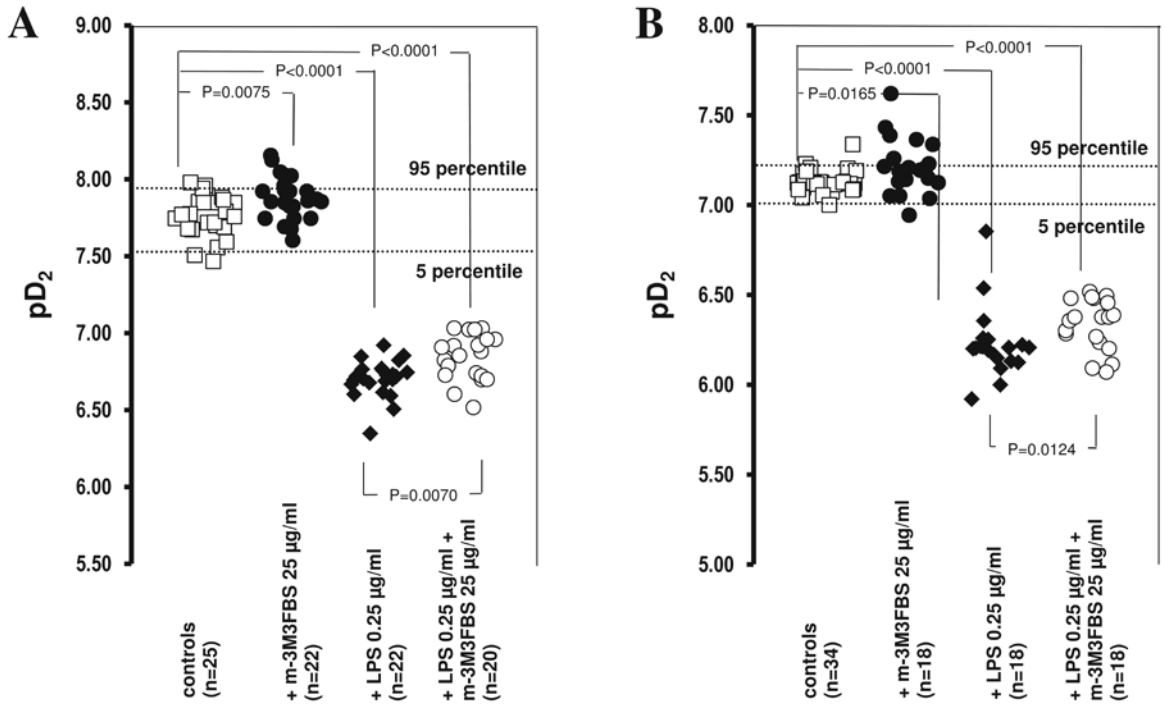

Figure 1. Impact of m-3M3FBS on pD_2 values for (A) AVP- or (B) PHE-induced contraction of tail arteries of Wistar rats with or without pre-treatment with LPS. m-3M3FBS, 2,4,6-trimethyl-*N*-[3-(trifluoromethyl)phenyl]benzenesulfonamide; AVP, arginine-vasopressin; PHE, phenylephrine; LPS, lipopolysaccharides; pD_2, negative logarithm of the half maximal effect concentration (EC_{50}).

pressure following stimulation of calcium influx from intracellular (phase 1) and extracellular (phase 2) calcium stores was measured and compared against the control cells and the cells treated with LPS. In the presence of m-3M3FBS, a significant increase (P<0.05) in perfusion pressure associated with calcium influx induced by PHE and AVP from the intra- and extracellular space was found compared with the controls. Furthermore, a significant increase in perfusion pressure associated with calcium influx in the presence of m-3M3FBS was detected in arteries pre-treated with LPS (P<0.0001; Fig. 2, Table II).

Discussion

Early studies have investigated the effects of m-3M3FBS on the concentration of secondary messengers, including DAG and IP_3, to confirm its stimulatory effect on PLC (8,9). In addition, the effects of m-3M3FBS on the concentration of calcium ions in the cytoplasm have been assessed without analysing its association with the tissue response (6,9). Therefore, in the present study, physiological and pharmacometric methods were used to evaluate the role of such biochemical changes on the response

Table II. Impact of m-3M3FBS on maximal perfusion pressure for AVP and PLP-induced contraction activated by calcium influx from intracellular (phase 1) and extracellular calcium stores (phase 2), with or without LPS.

		Intracellular calcium Phase 1	Extracellular calcium Phase 2
Treatment	n	Perfusion pressure [mmHg]	Perfusion pressure [mmHg]
AVP	25	61.2±6.7	103.9±6.2
AVP + m-3M3FBS	18	69.3±6.5[b]	118.7±7.6[c]
AVP + LPS	18	18.8±6.5	33.6±6.3
AVP + LPS + m-3M3FBS	18	38.6±7.1[c]	68.2±6.3[c]
PHE	25	57.2±6.9	94.9±7.7
PHE + m-3M3FBS	22	66.3±6.4[a]	114.2±7.0[c]
PHE + LPS	22	19.3±6.3	36.9±5.8
PHE + LPS + m-3M3FBS	20	25.2±6.7[c]	54.0±5.1[c]

[a]P<0.05, [b]P<0.0005, [c]P<0.0001, vs. controls. Values are expressed as the mean ± standard deviation. n, number of concentration-response curves used for calculations; AVP, arginine-vasopressin; m-3M3FBS, 2,4,6-trimethyl-*N*-[3-(trifluoromethyl)phenyl]benzenesulfonamide; PHE, phenylephrine; LPS, lipopolysaccharides; SD, standard deviation.

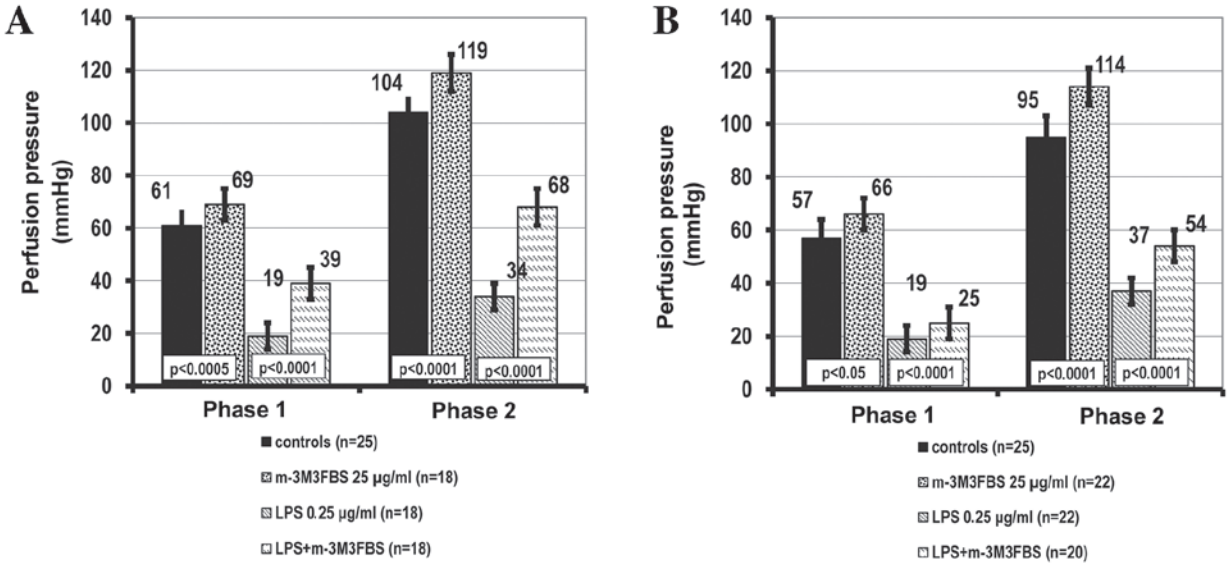

Figure 2. Impact of m-3M3FBS on maximal perfusion pressure in tail arteries of Wistar rats with (A) AVP- and (B) PHE-induced contraction activated by calcium influx from intracellular (phase 1) and extracellular (phase 2) calcium stores, with or without pre-treatment with LPS. m-3M3FBS, 2,4,6-trimethyl-*N*-[3-(trifluoromethyl)phenyl]benzenesulfonamide; AVP, arginine-vasopressin; PHE, phenylephreine.

of vascular smooth muscle. Activation of PLC is a key event in numerous metabotropic receptors, the physiological response of which was assessed in the present study. Inhibition of PLC function leads to a reduction in the calcium ion concentration in the cytoplasm of cells activated by the stimulation of α1-adrenergic receptors (13,18,19), endothelin receptor type A (20) and angiotensin II receptor type-1 (21). Furthermore, it has been demonstrated that the increase in cytoplasmic calcium concentration induced by PLC is significant at the molecular level and able to induce a significant physiological reponse (14).

The production of NO via induction of NOS-2 and -3 may be responsible for the dysfunction of vascular smooth muscle in endotoxemia (22) by decreasing the receptor reserve (2,3). Results of experiments performed in the presence of NOS inhibitors (iNOS) suggest that this peripheral effect may be partially reversible. Induction of iNOS in the rat aorta has been observed 60 min following LPS administration (23). Olsson *et al* (24) have demonstrated that production of NO and cyclic guanosine monophosphate in the rat urinary bladder is increased following intraperitoneal injection of *Escherichia coli* LPS. By contrast, MacMicking *et al* (25) identified a decreased mortality rate of iNOS-deficient mice. Treatment with iNOS increases receptor reserve and perfusion pressure in various experimental models, and may thus be a possible method of pharmacological intervention for early sepsis (26). Other mechanisms of increasing smooth muscle function, associated with increases in the function of various post-receptor enzymes, were analyzed in previous experimental studies (13-15). PLC is present in a number of different tissues and thus, there is no selectivity in enzyme stimulation; furthermore, in the presence of PLC activators, peripheral and

central effects may occur (13). The potential protective effect of m-3M3FBS against sepsis has been previously suggested by Kim *et al* (15). PLC activation enhanced the bactericidal activity and hydrogen peroxide production of mouse neutrophils and also enhanced the production of IFN-γ and IL-12, while inhibiting pro-septic TNF-α and IL-1β production in mice subjected to cecal ligation and puncture (15). In a second model of sepsis, PLC activation inhibited the production of TNF-α and IL-1β following systemic LPS challenge (24,26). It was concluded that stimulation of the central signal-transducing enzyme PLC by m-3M3FBS may reverse the progression of toxic shock by triggering multiple protective downstream signaling pathways to maintain organ function and leukocyte survival, and enhance microbial killing (24,26). The present study suggests that this central mechanism of possible protection is expanded by the peripheral mechanism of improving vascular function. The increase in contractility of vascular smooth muscle cells in the presence of m-3M3FBS has also been reported in LPS pre-treated tissues, by one of the authors of the present study in their habilitation thesis (14). The present study was performed to confirm these results. The results of the present study indicated that the peripheral protective effect in LPS-pre-treated arteries is the result of increased calcium influx from intra- and extracellular calcium stores.

In conclusion, the results of the present study suggested that m-3M3FBS significantly increased the response of vascular smooth muscle pre-treated with LPS by increasing calcium influx from intra- and extracellular calcium stores. Further investigation into this mechanism is required and may enable m-3M3FBS to be developed as a therapeutic to treat sepsis.

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