Regulation of cardiovascular GPCR signalling

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LPS differentially affects vasoconstrictor responses: a potential role for RGS16?

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Chapter 6

ABSTRACT

The profound hypotension in septic shock patients is difficult to treat as it is accompanied by depressed constrictor responses to $\alpha_1$-adrenoceptor agonists. Bacterial lipopolysaccharide (LPS) is the main trigger for most of the cardiovascular alterations occurring in septic shock. In this study we investigated the effects of LPS exposure on vascular contractility in general and the role of Regulator of G protein Signalling (RGS) proteins in the LPS-induced vascular alterations.

Exposure of rat aortic rings to various LPS concentrations (3, 10, 30 $\mu$g/ml) for 22 h differentially affected agonist-induced contractile responses at four distinct G-protein coupled receptors ($\alpha_1$-adrenoceptors, angiotensin II, serotonin and endothelin-1 receptors). While the endothelin-1-induced contraction was unaffected by LPS pre-treatment, phenylephrine- and angiotensin II-induced contraction were significantly reduced whereas serotonin-induced contraction was significantly enhanced. Concomitantly, LPS treatment increased the RGS16 mRNA expression both in aortic rings and cultured vascular smooth muscle cells (VSMCs) but not that of RGS2, RGS3, RGS4 or RGS5. The significant increase in RGS16 mRNA expression in VSMCs by LPS was time- and concentration-dependent but independent of increased inducible NO synthase (iNOS) activity. The changes in RGS16 mRNA might contribute to the differential regulation of the contractile responses to vasoconstrictors upon LPS exposure.
INTRODUCTION

Bacterial endotoxin or lipopolysaccharide (LPS) is the main trigger for most of the cardiovascular alterations occurring in septic shock (Parrillo et al., 1990) including a decreased responsiveness to vasoconstrictors. LPS induces, most likely via stimulation of the Toll-like receptor 4 (TLR4) on immune cells, a strong immune response by promoting the release of pro-inflammatory cytokines and mediators like nitric oxide (NO), platelet activating factor and leukotrienes resulting in inflammation and profound hypotension (Opal, 2007). The increased NO production leads to vasodilatation and hypo-responsiveness to vasoconstrictors (Murray et al., 2000). However, inhibition of NO production alone is not sufficient to fully restore the systemic vascular resistance (Wylam et al., 2001), indicating the involvement of other factors in the hypo-responsiveness.

Toll-like receptors, including TLR4, are not only expressed on immune cells but also on vascular smooth muscle cells (VSMCs) and endothelial cells (Dauphinee and Karsan, 2006; Shuang et al., 2007; Yang et al., 2005). Therefore, LPS can also directly affect vasoconstrictor mechanisms, e.g. by altering the expression level of vascular inducible NO synthase (iNOS) and vasoconstrictor receptors or proteins involved in the downstream signalling of these receptors (Matsuda and Hattori, 2007). Patten et al. (Patten et al., 2002) showed that LPS-induced cardiac failure is associated with alterations in regulator of G protein signalling (RGS) proteins, especially in RGS4 and RGS16 expression. RGS proteins are a family of intracellular proteins that are able to fine-tune G protein-coupled receptor (GPCR) signalling either by acting as GTPase activating proteins for the Gα subunits or by providing a signalling network through protein-protein interactions with various signalling proteins (for review see Hendriks-Balk et al., 2008; Hollinger and Hepler, 2002; Ross and Wilkie, 2000; Siderovski and Willard, 2005). Most of the RGS proteins interact with members of the Gαi and Gαq subfamily. In the cardiovascular system several RGS proteins are expressed and only for a small subset a physiological role has been established (Hendriks-Balk et al., 2008; Wieland et al., 2007; Wieland and Mittmann, 2003). In blood vessels, for instance RGS2 is known to play an important role in maintaining the vascular tone. Cells from hypertensive patients show a decrease in RGS2 expression levels (Semplicini et al., 2006) and RGS2 knockout mice show a hypertensive phenotype and a prolonged vasoconstriction (Heximer et al., 2003). In contrast, increased RGS2 expression levels are found in patients with Bartter’s/Gitelman’s syndrome which is characterized by hypotension and several other defects (Calo et al., 2008; Calo et al., 2004).

In this study we investigated whether LPS may affect the signalling of vasoconstrictors, such as phenylephrine, angiotensin II, serotonin and endothelin-1, differentially in isolated rat aortic rings and whether these changes are associated with changes in RGS expression.
MATERIALS AND METHODS

Materials
Collagenase I (crude), elastase type I, gelatin, trypsin inhibitor, bovine serum albumin, endothelin-1, serotonin, phenylephrine, N\textsuperscript{G}-nitro-L-arginine (L-NAME), N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), RNA later\textsuperscript{®} and LPS were from Sigma-Aldrich (Zwijndrecht, The Netherlands). Medium 199, penicillin/streptomycin, foetal bovine serum (FBS), trypsin/EDTA, phosphate buffered saline (PBS), Trizol reagent and DNAse I (Amplification grade) were obtained from Invitrogen (Breda, The Netherlands). iScript\textsuperscript{™}cDNA Synthesis kit, iQ\textsuperscript{™} SYBR\textsuperscript{®} Green Supermix and the Experion RNA StdSense Analysis Kit were purchased from Bio-Rad Laboratories BV (Veenendaal, The Netherlands). Primers were from Biolegio (Nijmegen, The Netherlands). Angiotensin II was obtained from Bachem (via Instruchemie, Delfzijl, The Netherlands).

Isolation and culture of vascular smooth muscle cells
The experimental protocol was approved by the Animal Ethical Committee of the University of Amsterdam, The Netherlands. Male Wistar rats (Charles River, Maastricht, The Netherlands) weighing 270-330 g were killed by an overdose of pentobarbital (100 mg/kg, i.p.) and their thoracic aortas were removed. The aortas were cleaned of fat and connective tissue under aseptic conditions and rinsed several times in Medium 199 containing penicillin (100 U/ml) and streptomycin (100 µg/ml). The cleaned vessels were incubated, shaking, at 30°C in 5 ml digestion medium (Medium 199 containing 1 mg/ml collagenase I, 0.1 mg/ml elastase, 0.5 mg/ml soybean trypsin inhibitor, 1 mg/ml BSA and penicillin/streptomycin) for 30 min before the adventitial layer was removed as an everted tube. The remaining tissue was rinsed with Medium 199 containing antibiotics and further incubated in 5 ml digestion medium at 37°C for 45 min. The partly digested tissue was then rinsed in Medium 199 containing antibiotics, cut into very small pieces and incubated for a further 2.5 h in 8 ml digestion medium at 37°C. After digestion, single cells were prepared by tituration and washed twice by resuspension in Medium 199 supplemented with 20% FBS and antibiotics. The isolated VSMCs were cultured in flasks coated with 0.1% gelatin in Medium 199 supplemented with 20% FBS and antibiotics for one week with changing the medium every 2-3 days. These VSMCs were defined as passage 0. After first passaging using trypsin/EDTA, VSMCs were cultured in Medium 199 containing 10% FBS and antibiotics. They were cultured until confluency with change of medium every 2-3 days before the cells were split 1:2. When passing the VSMCs the passage number was increased by one. All cells were cultured in a humidified incubator with 95%O\textsubscript{2}/5%CO\textsubscript{2} at 37°C.
LPS exposure and contraction of aortic rings

The cleaned vessel was dissected in rings of equal size (length ~0.3 mm). Aortic rings were washed twice in Medium199 containing penicillin (100 U/ml) and streptomycin (100 µg/ml) and incubated with various concentrations LPS in Medium199 with antibiotics for the indicated time period in a humidified incubator with 95%O₂/5%CO₂ at 37°C. The aortic rings were mounted between two stainless steel hooks into organ baths containing 5 ml Tyrode’s solution (118.5 mM NaCl, 4.7 mM KCl, 25.0 mM NaHCO₃, 1.2 mM MgSO₄·7H₂O, 0.025 mM Na₄EDTA, 1.8 mM CaCl₂, 1.2 mM KH₂PO₄ and 5.5 mM glucose) at 37 °C. The buffer was continuously gassed with a mixture of 95% O₂ and 5% CO₂ (carbogen) (pH 7.4). Each preparation was fixed, via a silk thread, to an isometric force transducer and force was recorded via a PowerLab data acquisition system (A.D. Instruments, Castle Hill, Australia). The aortic rings were equilibrated for 1 h at a resting tension of 10 mN, which was maintained throughout the experiment. The aortic rings were primed by exposing them four times to a depolarizing 40 mM KCl solution for 10 min. After each depolarization the buffer was exchanged and the aortic rings were allowed to equilibrate for 30 min. Between the third and the fourth KCl-induced vasoconstriction, the endothelial function of the vessel rings was tested by applying a precontraction with phenylephrine (1 µM) followed by a single concentration (1 µM) of methacholine. Aortic rings with a methacholine-induced relaxation greater than or equal to 80% were regarded as rings with an intact endothelium and included in the experiments. A cumulative concentration response curve for several vasoconstrictors was constructed 30 min after the fourth KCl-induced contraction. In experiments using angiotensin II, L-NNA (0.1 mM) was added to the buffer 20 min prior to the addition of the angiotensin II to facilitate contraction by angiotensin II.

LPS exposure followed by RNA isolation and cDNA synthesis

The cleaned vessel was dissected in rings of equal size (length ~0.3 mm). Aortic rings were washed twice in Medium199 containing penicillin and streptomycin and incubated with 1 µg/ml LPS for 2 h. The aortic rings were stored in RNAlater at -20°C for at least 24 h, cut into very small pieces and lysed in Trizol. After at least one day storage in Trizol, the tissue was homogenized, shaken vigorously for 30 min and centrifuged before RNA isolation.

VSMCs of passage 4-6 were cultured into 6 cm culture dishes (coated with 0.1% gelatine) until confluency. After overnight serum starvation the cells were exposed to various concentrations LPS for the indicated time periods, washed twice with PBS and lysed with 2 ml Trizol. For aortic ring or isolated cell samples total RNA was isolated according to the manufacturer’s protocol with minor changes, using a second chloroform extraction to remove traces of phenol in the aqueous phase, a high salt solution (0.8 M sodium citrate, 1.2 M NaCl) together with isopropanol to precipitate RNA and a second wash of the RNA pellet with 75% ethanol.
RNA purity was verified by electrophoresis (Experion, Biorad Laboratories, Veenendaal, The Netherlands) and the RNA concentration was determined by spectrophotometry (Nanodrop, Isogen Life Science, IJsselstein, The Netherlands). To eliminate genomic DNA contamination 1 µg of total RNA was treated with 1 µl DNase I, Amp Grade, according to the manufacturer’s protocol. The RNA sample was split into a sample for cDNA synthesis and a control sample. cDNA was synthesized by reverse transcription using the iScript cDNA Synthesis kit according to the manufacturer’s protocol. A control sample, in which no cDNA was synthesized, was made for each sample to check for the presence of genomic DNA. The cDNA of 1 µg RNA was diluted 1:50 for use in real-time quantitative PCR.

**Primer design and real-time quantitative PCR (QPCR)**

Oligonucleotide primers were designed using the D-LUX designer software (Invitrogen), based on sequences from the GenBank database (Table 1). Each primer pair was tested for selectivity using melt curve analysis of a gradient PCR, and for sensitivity and efficiency using a standard curve of template DNA. Constitutively expressed elongation factor-1 (EF-1) and p0 ribosomal protein were selected as endogenous controls to correct for potential variation in RNA loading (for criteria see (Hendriks-Balk et al., 2007)).

Relative quantification of mRNA was performed on a MyiQ Single-Color Real-Time PCR Detection System (Biorad Laboratories) following the thermal protocol: 95°C for 3 min to denature, 40 cycles (for RGS16 55 cycles) at 95°C for 10 s followed by 60°C for 45 s for an-

<table>
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Table 1. Oligonucleotide primers used for real-time quantitative polymerase chain reaction. Non-capital letters indicate that these nucleotides are added to form a hairpin.
nealing and extension. The final reaction mixture of 15 µl consisted of diluted cDNA, 1x iQ SYBR Green Supermix, 200 nM forward primer and 200 nM reverse primer. All the reactions were performed in 96-well plates, in duplicate. Controls for genomic DNA were included for each cDNA sample and also a negative control containing only both primers and the iQ SYBR Green Supermix. All data were corrected for difference in RNA loading using the expression of EF-1 and p0 ribosomal protein, which did not change upon LPS exposure.

Data analysis

QPCR data were analysed using the Gene expression Macro software (Biorad Laboratories) based on algorithms of Vandesompele et al. (Vandesompele et al., 2002). The data are expressed as mean relative expression ± standard error of the mean (S.E.M.) of independent measurements on VSMCs isolated from different rat aortas and related to the expression level in the unstimulated VSMCs. Statistical analysis of the relative expression was performed with a one-way ANOVA, followed by a multiple comparison post-hoc test (Student-Newman-Keuls test) using GraphPad Instat (GraphPad Software, San Diego, CA, USA). Organ bath data are expressed as % of the fourth KCl-induced contraction ± S.E.M. of independent measurements on aortic rings from different rats. Statistical analysis of the organ bath data was performed with a one-way ANOVA with a Dunnett’s correction. A P<0.05 was considered as statistically significant.

RESULTS

Effect of LPS exposure on vascular contractile responses

Exposure of aortic rings to various concentrations of LPS for 22 h had no effect on KCl (40mM)-induced contractile force (9.3 ± 0.4, 9.1 ± 0.3 and 9.3 ± 0.2 mN for 3, 10 and 30 μg/ml LPS exposure, respectively, versus 8.9 ± 0.2 mN for control, n>18), indicating that the contractile machinery is not affected by LPS exposure.

After 22 h culturing in the absence of LPS, phenylephrine increased the force of contraction in the aortic rings with a pEC\textsubscript{50} of 6.9 ± 0.1 and a maximum of 81 ± 4 % (n=12) (Fig. 1A). A significant rightward shift of the concentration response curve of phenylephrine was found after 22 h exposure to 3 and 10 μg/ml LPS (pEC\textsubscript{50} of 6.6 ± 0.1 and 6.4 ± 0.1, respectively, n=6-10, P<0.05 versus control), whereas maximal phenylephrine-induced contraction was only marginally affected (88 ± 7 and 60 ± 11 %, respectively, n=6-10) (Fig. 1A). Interestingly, exposure to 30 μg/ml LPS did not shift the curve any further to the right, but rather shifted the curve back (pEC\textsubscript{50} of 6.8 ± 0.1, n=7) to the untreated control, indicating a bell-shaped relationship between the LPS concentration and its effect on the contractile response (Inset
Fig. 1A). Similarly, angiotensin II-induced contraction was attenuated by LPS exposure (Fig. 1B). A concentration response curve for angiotensin II-induced contraction could only be constructed in aortic rings that were not exposed to LPS (pEC$_{50}$ of 8.3 ± 0.1 and E$_{max}$ 67 ± 6 %, n=5). Because of the increased tachyphylaxis in LPS-treated preparations only one concentration of angiotensin II (3*10$^{-8}$ M) was tested, and the contraction was significantly decreased after exposure to 3, 10 and 30 µg/ml LPS (5 ± 4, 3 ± 1, 18 ± 17 %, respectively versus 57 ± 4 % without LPS, n=3-5, P<0.05) (Fig. 1B).

In contrast, LPS exposure had no significant effect on contraction induced by endothelin-1 (Fig. 1C). Interestingly, in contrast to phenylephrine and angiotensin II, serotonin-induced contraction was enhanced after LPS exposure. The concentration response curve showed a significant leftward shift after exposure to 10 and 30 µg/ml LPS (pEC$_{50}$ of 5.4 ± 0.1 and 5.8 ± 0.1, respectively, versus 5.1 ± 0.1 without LPS, n=6-7, P<0.05), and also a significant increase in maximal contraction after exposure to 30 µg/ml LPS (102 ± 2 % versus 76 ± 5 % without LPS, n=6-7, P<0.05) (Fig. 1D).

![Graphs A, B, C, D](image)

**Fig. 1.** Contractile responses in rat aortic rings to phenylephrine (A), angiotensin II (B), endothelin-1 (C) and serotonin (D) after exposure to different concentrations LPS for 22 h. Inset in A: log EC$_{50}$ values of the contractile response to phenylephrine after LPS exposure. For angiotensin II-induced constriction, aortic rings were preincubated with L-NNA (0.1 mM) for 20 min. Data are expressed as means ± S.E.M., n=3-13.
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Fig. 2. Time-dependency of LPS-induced regulation of mRNA expression levels of RGS proteins upon 10 μg/ml LPS exposure. Data are expressed relative to unexposed VSMCs at starting point (t=0 hr), which was set arbitrarily to one in each experiment. Mean raw Ct values at starting point are: 30 ± 0.5 (RGS2), 28 ± 0.4 (RGS3), 28 ± 1 (RGS4), 30 ± 1 (RGS5) and 34 ± 2 (RGS16). Relative expression levels are presented as mean ± S.E.M., n=3-6; *P<0.05. Note differences in y-axis scale.

Effect of LPS exposure on RGS mRNA expression in VSMCs

We determined mRNA expression of iNOS, RGS2, RGS3, RGS4 and RGS5 in cultured VSMCs after several time periods of LPS (10 μg/ml) exposure. While the expression of iNOS was virtually absent in unstimulated VSMCs and VSMCs exposed to vehicle, it was highly increased after 2, 6 and 22 h of LPS exposure with highest expression after 6 h (raw Ct values: from >37 in unstimulated and vehicle-stimulated VSMCs to 28 ± 1 in VSMCs exposed to LPS for 6 h). The expression of RGS16 mRNA was significantly increased after 2 h of LPS exposure (Fig. 2). This increase of RGS16 mRNA expression however was transient as the levels dropped to basal level after 22 h. No significant time-dependent alterations in RGS2, RGS3, RGS4 and RGS5 mRNA expression were found after LPS exposure (Fig. 2).

In addition to the time-dependent effects of LPS, induction of iNOS and RGS16 mRNA expression after 2 h was dependent on LPS concentration. Raw Ct values of iNOS expression dropped from 39 ± 1 to 30 ± 0.4 at increasing LPS concentrations from 0 μg/ml to 30 μg/ml, respectively, indicating a large increase in iNOS expression. Exposure (2 h) to different LPS concentrations had no effect on RGS5 mRNA expression (Fig. 3A). However, RGS16 mRNA expression was increased at increasing LPS concentrations (Fig. 3B). In addition, RGS16 mRNA was up-regulated 4-fold in aortic rings after 2 h exposure with 1 μg/ml LPS (inset Fig. 3B). Expression levels of RGS2, RGS3, RGS4 and RGS5 mRNA were not significantly changed in aortic rings (data not shown).
To evaluate whether the transient changes in RGS16 mRNA expression were dependent on the increased NO production by iNOS, VSMCs were pre-incubated 30 min with 0.1 mM L-NAME prior to LPS exposure (10 μg/ml, 2 h). Inhibition of NO production by L-NAME had no effect on the LPS-induced RGS16 expression (Fig. 4).

**Fig. 3.** mRNA expression levels of RGS5 (A) and RGS16 (B) in VSMCs after 2 h exposure to different concentrations of LPS. Inset in B: mRNA expression level of RGS16 in aortic rings after 2 h exposure to 1 μg/ml LPS. Data are expressed relative to unexposed VSMCs at starting point (t=0 hr) or to vehicle exposure in aortic rings, which was set arbitrarily to one in each experiment. Mean raw Ct values at starting point are: 28 ± 1 (RGS5), 34 ± 1 (RGS16, VSMCs) and 30 ± 1 (RGS16, aortic rings). Relative expression levels are presented as mean ± S.E.M., n=3-5, *P<0.05 compared to vehicle exposure for 2 h.

**Fig. 4.** Effect of L-NAME (0.1 mM) pre-incubation on the up-regulation of RGS16 mRNA expression induced by 2 h exposure to 10 μg/ml LPS. Data are expressed relative to unexposed VSMCs at starting point (t=0 hr), which was set arbitrarily to one in each experiment. Mean raw Ct value at starting point is: 37 ± 1. Relative expression levels are presented as mean ± S.E.M., n=4-5.

**Effect of L-NAME on LPS-mediated RGS16 mRNA up-regulation**

To evaluate whether the transient changes in RGS16 mRNA expression were dependent on the increased NO production by iNOS, VSMCs were pre-incubated 30 min with 0.1 mM L-NAME prior to LPS exposure (10 μg/ml, 2 h). Inhibition of NO production by L-NAME had no effect on the LPS-induced RGS16 expression (Fig. 4).
DISCUSSION

Bacterial endotoxin/LPS-induced septic shock is characterized by a profound hypotension, eventually leading to organ hypoperfusion and ultimately organ failure. Remarkably, due to hypo-responsiveness to vasoconstrictors, the hypotension is difficult to overcome by administration of noradrenaline (Groeneveld et al., 1986). Since it is not that well investigated yet whether the hypo-responsiveness observed is \( \alpha_1 \)-adrenoceptor-specific or whether G protein-coupled receptor-mediated vasoconstriction is decreased in general, we have investigated the overall effect of LPS treatment on G protein-coupled receptor-mediated contraction in rat aortic rings.

In this study we show that all four GPCRs investigated are affected differentially by LPS exposure. We show in this study that LPS (3 and 10 \( \mu \)g/ml) impaired contractility of the rat aorta in response to phenylephrine, an \( \alpha_1 \)-adrenoceptor agonist. Unexpectedly, a further increase in LPS concentration did not further decrease the phenylephrine-induced contraction but rather shifted the concentration response curve back to the untreated control situation, suggesting a bell-shaped relation.

Due to pronounced tachyphylaxis (Gruetter et al., 1987) and/or the LPS-induced changes therein we were not able to construct a concentration response curve for the angiotensin II-induced contraction in the LPS-treated aortic rings, whereas it could be constructed in the untreated aortic rings. Application of a high concentration of angiotensin II revealed that the aortic rings exposed to LPS for 22 h are still able to contract albeit with significantly decreased efficacy.

Interestingly, in contrast to the decreased vascular reactivity towards phenylephrine and angiotensin II, LPS exposure seemed to increase the responsiveness to serotonin in rat aorta, in potency as well as in maximal contraction. In agreement with our results, Wylam et al. (Wylam et al., 2001) also showed an increase in maximal serotonin-induced contraction in intact rat aortic rings after LPS exposure for 20 h. A similar response was observed in rat cerebral arteries where the serotonin-induced contraction was increased after exposure to LPS for 1-4 h and returned to the control situation again after longer LPS exposure times (Hernanz et al., 2003).

In contrast to the affected vascular reactivity towards phenylephrine, angiotensin II and serotonin, LPS has no major effects on the contraction of the rat aorta mediated by endothelin-1, one of the most potent vasoconstrictors. This is in contrast with a study performed by Ishimaru et al. (Ishimaru et al., 2001) where they measured the effect of exogenous endothelin-1 on the mean arterial pressure in anesthetized rats. They showed that LPS treatment decreased the effect of endothelin-1 on the mean arterial pressure (Ishimaru et al., 2001). This difference in findings might be explained by the cardiac or central effects of LPS as they infused the rat with LPS whereas we only treated the isolated aortic rings. In addition, in the
aforementioned study rats were exposed to LPS for 2 h whereas we used an exposure of 22 h to LPS in aortic rings.

Our study clearly shows that LPS differentially affect the signalling via four distinct GPCRs involved in smooth muscle contraction. We cannot exclude a reduction in $\alpha_1$-adrenoceptor expression, as was demonstrated in the rat liver after chronic LPS infusion (Roth and Spitzer, 1987), or an up-regulation of the serotonin receptor involved in serotonin-mediated contraction. However, other studies indicate that the angiotensin II receptor expression is up-regulated in VSMCs (Burnier et al., 1995). This cannot explain our observations that the angiotensin II-mediated contraction is reduced upon LPS exposure. Overall, our results support the hypothesis that beside NO and alterations in receptor expression other factors, are involved in the effects observed upon LPS exposure.

Previous studies have suggested a role for RGS4 and RGS16 in the early onset of cardiac failure during septic shock (Patten et al., 2002). Probably, alterations in RGS protein expression also play a role in the hypotension associated with LPS treatment and the hyporesponsiveness of several vasoconstrictors. In this respect, it is interesting to mention that Rgs2 knockout mice were found to be hypertensive and show a prolonged vasoconstriction upon angiotensin II stimulation (Heximer et al., 2003). Furthermore, cells from hypertensive patients show a decrease in RGS2 protein expression and an increased responsiveness to angiotensin II (Semplicini et al., 2006) whereas an increase in RGS2 expression has been found in cells from patients with Bartter’s/Gitelman’s syndrome, which is characterized by hypotension and several other defects (Calo et al., 2008; Calo et al., 2004). Besides RGS2 also other RGS proteins are suggested to be involved in blood pressure regulation. For instance, RGS5 is suggested to play a role in vasodilatation as Rgs5 knockout mice are hypertensive (Cho et al., 2008). Because of aforementioned we investigated the effect of LPS exposure on mRNA expression of a selection of RGS proteins, including RGS2, RGS4, RGS5 and RGS16, in VSMCs and aortic rings.

We demonstrate that the mRNA expression of RGS16 in cultured VSMCs is time- and concentration-dependently regulated upon LPS exposure. After 2 h of LPS exposure the RGS16 mRNA expression is increased and returned to basal levels after 22 h of exposure. The expression levels of RGS2, RGS3, RGS4 and RGS5 were, however, not altered. The results observed in VSMCs were confirmed in rat aortic rings. Again, mRNA expression of RGS16 was up-regulated by LPS exposure whereas RGS2, RGS3, RGS4 and RGS5 expression levels were also unaffected. These data are in line with the reported transient increase in RGS16 mRNA and protein expression in rat hearts and cultured neonatal cardiomyocytes after LPS treatment (Patten et al., 2002). Also in pig heart and aorta an up-regulation of RGS16 mRNA expression upon LPS exposure has been described (Panetta et al., 1999).

It is well known that LPS exposure induces the expression of iNOS in endothelial as well as in VSMCs (Dauphinee and Karsan, 2006; Hattori et al., 2003). The resulting increase in NO production is a major pathway whereby LPS reduces vascular responsiveness. Since NO was
described to regulate several transcription factors and hence gene expression (Contestabile, 2008), we investigated whether the increased RGS16 expression was caused by increased NO production due to LPS treatment. Inhibition of NOS activity with L-NAME did not attenuate the effect of LPS on RGS16 mRNA expression suggesting that the increased RGS16 mRNA expression is independent of the enhanced NO production by LPS.

Our study, together with previous studies in myocardial tissue, clearly indicates regulation of the RGS16 expression upon LPS exposure. However, conclusive evidence clearly linking increased RGS16 expression to the altered vascular reactivity towards vasoconstrictors upon LPS exposure is still missing.

In conclusion, our data indicate that LPS differentially regulates the contractile responses to vasoconstrictors as measured in aortic rings. In addition, our results show for the first time that the mRNA expression of RGS16 in VSMCs is time- and concentration-dependently up-regulated by LPS. The ability to up-regulate the expression of RGS16 is independent of the induction of NOS activity. This change in RGS16 expression might be one of the candidates explaining the NO-independent part of the altered vascular response to vasoconstrictors upon LPS exposure.
REFERENCES


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