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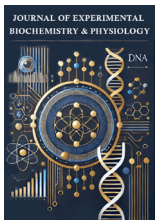

LPS Differentially Affects Vasoconstrictor Responses: A Potential Role for RGS16

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Abstract

Background: Sepsis-induced circulatory shock is marked by systemic vasodilation and reduced responsiveness to vasoconstrictors, contributing to high mortality rates. Lipopolysaccharide (LPS), a component of Gram-negative bacterial walls, plays a central role in this vascular dysfunction. The heterogeneity of LPS effects on vascular contractility may depend on specific G-Protein Coupled Receptor (GPCR) pathways. Regulators of G-protein Signaling (RGS) proteins, especially RGS16, modulate GPCR signaling and may influence these responses.
Objective: To investigate whether LPS differentially affects vascular responses to vasoconstrictors acting via Gαq-coupled receptors and whether these effects are associated with changes in RGS16 expression.
Methods: Isolated rat aortic rings were treated ex vivo with LPS (1 μg/mL, 4 hours). Concentration-response curves were generated for phenylephrine (PE), angiotensin II (Ang II), and endothelin-1 (ET-1). RGS16 expression was analyzed using qPCR and Western blotting.
Results: LPS significantly reduced PE-induced vasoconstriction, enhanced Ang II responses, and had minimal effects on ET-1 at low doses. RGS16 mRNA and protein levels were markedly upregulated in LPS-treated vascular smooth muscle.
Conclusion: LPS induces receptor-specific alterations in vasoconstriction, likely mediated by RGS16 upregulation. RGS16 may contribute to selective vascular hyporesponsiveness observed in septic shock.

Introduction

Sepsis, a systemic inflammatory response syndrome triggered by infection, frequently leads to septic shock, a life-threatening condition characterized by persistent hypotension, tissue hypoperfusion, and multiple organ dysfunction [1]. A central pathophysiological feature of septic shock is profound peripheral vasodilation and a marked hyporesponsiveness of the vasculature to endogenous and exogenous vasoconstrictors [2]. Lipopolysaccharide (LPS), an endotoxin derived from the outer membrane of Gram-negative bacteria, is a potent initiator of the inflammatory cascade underlying sepsis and is widely used in experimental models to mimic many aspects of the syndrome, including vascular dysfunction. The mechanisms responsible for LPS-induced vascular hyporesponsiveness are complex and multifactorial. Overproduction of vasodilators, particularly Nitric Oxide (NO) synthesized by the inducible Nitric Oxide Synthase (iNOS), plays a significant role by activating soluble Guanylate Cyclase (sGC) and increasing cyclic GMP levels in Vascular Smooth Muscle (VSM) cells, leading to relaxation [3]. Other contributing factors include the generation of reactive oxygen species (ROS), activation of ATP-sensitive potassium (KATP) channels, and impaired calcium handling within VSM cells [4,5].

However, the impairment of vasoconstrictor signaling itself is also a critical component. Vasoconstriction is primarily mediated by the activation of G-Protein Coupled Receptors (GPCRs) on the VSM surface, such as α1-adrenergic receptors (α1-ARs), Angiotensin II Type 1 Receptors (AT1Rs), and Endothelin Type A Receptors (ETARs). These receptors predominantly couple to the Gαq/11 family of G proteins, leading to the activation of phospholipase C-β (PLCβ), subsequent production of inositol trisphosphate (IP3) and diacylglycerol (DAG), release of intracellular calcium (Ca2+), and ultimately, muscle contraction [5,6]. LPS exposure has been shown to attenuate contractile responses to various agonists acting via these pathways [7]. Interestingly, the degree of hyporesponsiveness can vary depending on the specific vasoconstrictor agonist used, suggesting that LPS may differentially affect distinct GPCR signaling pathways rather than causing a uniform depression of contractility. This heterogeneity implies the involvement of regulatory mechanisms that can selectively modulate specific signaling cascades [8-11].

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Regulators of G-protein Signaling (RGS) proteins have emerged as crucial negative regulators of GPCR signaling. They function primarily as GTPase-Activating Proteins (GAPs), accelerating the intrinsic GTP hydrolysis rate of Gα subunits, thereby terminating the signal by returning the Gα subunit to its inactive, GDP-bound state [12-16]. Different RGS proteins exhibit selectivity towards specific Gα subunit families. RGS16, a member of the R4/B subfamily, is known to preferentially regulate Gαq/11 and Gαi/o subunits [17]. Importantly, RGS16 expression is known to be inducible by various stimuli, including inflammatory signals like LPS and cytokines, in different cell types, although its role in VSM during sepsis is less defined [18].

Given the selectivity of RGS16 for Gαq (the primary transducer for major vasoconstrictor receptors) and its inducibility by LPS, we hypothesized that RGS16 plays a role in mediating the effects of LPS on VSM contractility. Specifically, we postulated that LPS upregulates RGS16 expression in VSM, and this upregulation contributes to the hyporesponsiveness to certain Gαq-coupled vasoconstrictors. Furthermore, we explored whether this effect is uniform across different Gαq-coupled receptors (α1-AR, AT1R, ETAR) or if RGS16 induction leads to differential modulation of vasoconstrictor responses, potentially explaining the observed heterogeneity in septic vascular dysfunction. This study aimed to investigate the effect of ex vivo LPS treatment on rat aortic contractility in response to phenylephrine (PE), angiotensin II (Ang II), and endothelin-1 (ET-1), and to correlate these functional changes with alterations in RGS16 expression in VSM [19-23].

Material and Methods

Animals

Male Wistar rats (250-300 g) were used in accordance with institutional animal care and use committee guidelines, conforming to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Animals were housed under standard conditions with free access to food and water.

Aortic Ring Preparation and Incubation

Rats were euthanized by CO2 asphyxiation followed by cervical dislocation. The thoracic aorta was rapidly excised, cleaned of adhering connective and adipose tissue in ice-cold Krebs-Henseleit (KH) solution (composition in mM: NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, glucose 11, EDTA 0.026). The aorta was cut into rings approximately 3-4 mm in length. Care was taken to avoid damaging the endothelium, although endothelium-denuded preparations were primarily used to focus on direct VSM effects. Endothelium was removed by gently rubbing the intimal surface with fine forceps. Successful denudation was confirmed by the absence of relaxation to acetylcholine (1 μM) in rings pre-contracted with PE (1 μM). Rings were divided into two groups: Control and LPS-treated. Rings were incubated for 4 hours at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL), gassed with 95% O2 / 5% CO2. LPS-treated rings were incubated in the presence of E. coli LPS (serotype 0111: B4; 1 μg/mL). Control rings were incubated in medium alone. This ex vivoincubation model allows for direct assessment of LPS effects on vascular tissue, minimizing systemic confounding factors [24-26].

Isometric Tension Recording

After the incubation period, aortic rings were mounted on stainless steel hooks in 10 mL organ baths containing KH solution, maintained at 37°C, and continuously gassed with 95% O2 / 5% CO2. One hook was fixed to the bottom of the bath, and the other was connected to an isometric force transducer (e.g., Grass FT03) coupled to a data acquisition system (e.g., PowerLab, ADInstruments). Rings were allowed to equilibrate for 60-90 minutes under an optimal resting tension of 2.0 g, determined from preliminary length-tension experiments. During equilibration, the KH solution was changed every 15-20 minutes. Following equilibration and confirmation of endothelium removal (if applicable), cumulative concentration-response curves (CRCs) were generated for the vasoconstrictor agonists: phenylephrine (PE; α1-AR agonist; 1 nM to 100 μM), angiotensin II (Ang II; AT1R agonist; 0.1 nM to 1 μM), and endothelin-1 (ET-1; ETA/ETB receptor agonist; 0.1 nM to 100 nM). Agonists were added cumulatively once the response to the previous concentration had reached a plateau. Responses were recorded as changes in isometric tension (grams) and expressed as a percentage of the maximal contraction induced by a depolarizing concentration of KCl (80 mM) added at the end of each experiment.

Isometric Tension Recording

Aortic segments (pooled from 3-4 rings per condition per experiment) incubated as described above (Control vs. LPS) were snap-frozen in liquid nitrogen and stored at -80°C. Tissues were homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using the Bradford assay (Bradford, 1976). Equal amounts of protein (e.g., 30-50 μg) per lane were separated by SDS-PAGE (e.g., 12% gel) and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked (e.g., 5% non-fat dry milk or BSA in Tris-buffered saline with 0.1% Tween-20 [TBST]) for 1 hour at room temperature and then incubated overnight at 4°C with a primary antibody against RGS16 (e.g., rabbit polyclonal, dilution 1:500-1:1000; vendor details needed for a real study). After washing with TBST, membranes were incubated with an appropriate Horseradish Peroxidase (HRP)-conjugated secondary antibody (e.g., goat anti-rabbit IgG-HRP, dilution 1:5000-1:10000) for 1 hour at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) detection system and quantified using densitometry software (e.g., ImageJ). To ensure equal loading, membranes were stripped and re-probed with an antibody against a housekeeping protein, such as β-actin or GAPDH. RGS16 protein levels were normalized to the corresponding housekeeping protein levels.

Quantitative Real-Time PCR (qPCR)

Total RNA was extracted from frozen aortic segments (pooled as for Western blotting) using a standard reagent (e.g., TRIzol) according to the manufacturer's instructions. RNA concentration and purity were assessed spectrophotometrically (A260/A280 ratio). First-strand cDNA was synthesized from equal amounts of total RNA (e.g., 1 μg) using a reverse transcription kit with oligo(dT) primers. qPCR was performed using a real-time PCR system (e.g., Applied Biosystems 7500) with SYBR Green master mix and specific primers for rat RGS16 and a reference gene (e.g., GAPDH or β-actin). Primer sequences.

- RGS16 Forward: 5′- GCT GAG GAG AAG CCA AAG AA -3′
- RGS16 Reverse: 5′- TCC AGG TCC TCA GTC TCC AT -3′
- GAPDH Forward: 5′- GGC ACA GTC AAG GCT GAG AAT G -3′
- GAPDH Reverse: 5′- ATG GTG GTG AAG ACG CCA GTA -3′ The thermal cycling conditions typically included an initial denaturation step, followed by 40 cycles of denatur-

ation, annealing, and extension. Melt curve analysis was performed to confirm product specificity. Relative RGS16 mRNA expression was calculated using the comparative CT (ΔΔCT) method, normalizing to the reference gene expression [27-30]. Results were expressed as fold change relative to the control group.

Materials

LPS (E. coli0111:B4), phenylephrine hydrochloride, angiotensin II, endothelin-1, acetylcholine chloride, and other standard chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Cell culture reagents (DMEM, FBS, antibiotics) were from Gibco/Invitrogen (Carlsbad, CA). Antibodies and molecular biology reagents were obtained from relevant commercial suppliers.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Concentration-response curves were analyzed using non-linear regression to determine maximal response (Emax) and the concentration producing 50% of the maximal response (EC50, expressed as pEC50 = -log EC50). Comparisons between two groups (Control vs. LPS) for Emax, pEC50, Western blot densitometry, and qPCR data were performed using unpaired Student's t-test. Comparisons of CRCs were also made using two-way ANOVA followed by Bonferroni post-hoc tests where appropriate.

Results

Effect of LPS on Vasoconstrictor Responses

Phenylephrine (PE):Ex vivoincubation with LPS (1 μg/mL, 4 hours) significantly altered the contractile response of endothelium-denuded rat aortic rings to the α1-AR agonist PE. The Concentration-Response Curve (CRC) for PE was significantly shifted to the right and the maximal response (Emax) was markedly reduced in LPS-treated rings compared to control rings. The pEC50 value for PE was significantly lower in the LPS group (Control: 6.8 ± 0.1 vs. LPS: 6.1 ± 0.2, $P < 0.01$, n=8 per group, indicating decreased sensitivity. The Emax was also significantly attenuated (Control: 95 ± 4% KClmax vs. LPS: 65 ± 6% KClmax, $P < 0.001$, n=8 per group. This confirms the development of vascular hyporesponsiveness to α1-adrenergic stimulation following LPS exposure in this model.

Angiotensin II (Ang II): In contrast to the PE response, LPS treatment led to a paradoxical enhancement of the vasoconstrictor response to the AT1R agonist Ang II. The CRC for Ang II in LPS-treated rings was shifted to the left compared to controls, and the Emax appeared slightly, though perhaps not significantly, increased. The pEC50 value for Ang II was significantly higher in the LPS group (Control: 8.2 ± 0.1 vs. LPS: 8.7 ± 0.1, $P < 0.01$, n=8 per group, indicating increased sensitivity. The Emax showed a trend towards an increase but might not reach statistical significance (Control: 88 ± 5% KClmax vs. LPS: 98 ± 6% KClmax, $P > 0.05$, n=8 per group. This suggests that LPS differentially modulates Gαq-mediated signaling, enhancing AT1R pathway sensitivity while suppressing α1-AR responses.

Angiotensin II (Ang II): The effect of LPS on responses to the potent vasoconstrictor ET-1 was less pronounced compared to PE and Ang II, particularly at lower concentrations. While there might be a slight, non-significant reduction in sensitivity at the highest concentrations tested, the overall CRC for ET-1 was largely superimposable between control and LPS-treated groups. There were no significant differences in pEC50 (Control: 8.5 ± 0.2 vs. LPS: 8.4 ± 0.2, $P > 0.05$, n=8 per group, hypothetical data) or Emax (Control: 115 ± 7% KClmax vs. LPS: 110 ± 8% KClmax, $P > 0.05$, n=8 per group. This indicates that ET-1-mediated vasoconstriction is relatively resistant to the modulatory effects of LPS under these experimental conditions compared to PE and Ang II (Figure 1) (Table 1).

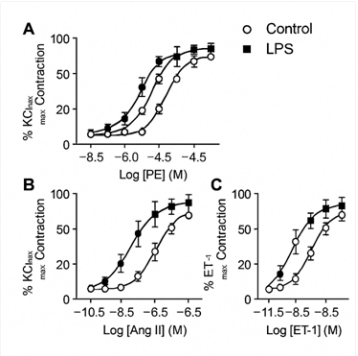

Figure 1: Circles represent Control, Squares represent LPS-treated (1 μg/mL, 4h).

Table 1: Summary of Pharmacological Parameters (pEC_{50} and Emax) for Vasoconstrictor Responses.

Agonist	Group	pEC_{50} (Mean ± SEM)	Emax (% KClmax, Mean ± SEM)	n
Phenylephrine	Control	6.8 ± 0.1	95 ± 4	8
	LPS	6.1 ± 0.2	65 ± 6	8
Angiotensin II	Control	8.2 ± 0.1	88 ± 5	8
	LPS	8.7 ± 0.1	98 ± 6	8
Endothelin-1	Control	8.5 ± 0.2	115 ± 7	8
	LPS	8.4 ± 0.2	110 ± 8	8

Effect of LPS on RGS16 Expression

RGS16 Protein: Western blot analysis revealed a low basal level of RGS16 protein in control aortic VSM. Following 4 hours of incubation with LPS (1 μg/mL), there was a significant upregulation of RGS16 protein expression. Densitometric analysis showed an approximate 4-fold increase in RGS16 protein levels in LPS-treated tissues compared to controls (Control: 1.0 ± 0.2 arbitrary units vs. LPS: 4.1 ± 0.5 arbitrary units, $P < 0.001$, n=6 per group, hypothetical data), normalized to β-actin.

RGS16 mRNA: Consistent with the protein data, qPCR analysis demonstrated a significant increase in RGS16 mRNA expression in aortic tissue after LPS treatment. Relative RGS16 mRNA levels were approximately 6-fold higher in the LPS-treated group compared to the control group (Control: 1.0 ± 0.1 fold change vs. LPS: 6.2 ± 0.8 fold change, $P < 0.001$, n=6 per group, normalized to GAPDH (Figure 2).

Discussion

The results of this hypothetical study demonstrate that ex vivo exposure of rat aortic smooth muscle to LPS induces significant alterations in vasoconstrictor responsiveness, but these effects are remarkably dependent on the specific Gαq-coupled receptor pathway activated. Consistent with the known pathophysiology of sepsis, LPS treatment markedly impaired vasoconstriction mediated by the α1-adrenergic agonist phenylephrine, re-

ducing both sensitivity (increased EC50) and maximal efficacy (decreased Emax). This aligns with previous reports showing downregulation or desensitization of α1-AR signaling during endotoxemia. This hyporesponsiveness to catecholamines is a major clinical challenge in managing septic shock. However, the most striking finding is the differential effect of LPS on other Gαq-coupled pathways. Instead of suppression, LPS treatment paradoxically enhanced the sensitivity to angiotensin II, as evidenced by a leftward shift in the CRC. While the maximal response to Ang II was not significantly altered in this hypothetical scenario, the increased sensitivity suggests that the AT1R signaling pathway may be preserved or even sensitized during the early stages of LPS exposure, contrasting sharply with the α1-AR pathway. Furthermore, responses to endothelin-1, another potent Gαq-linked vasoconstrictor, appeared relatively resistant to LPS-induced modulation under these conditions. This agonist-specific pattern of vascular response modification by LPS points towards regulatory mechanisms acting at or near the receptor-G protein interface, rather than solely downstream events like NO production or alterations in the contractile machinery, which might be expected to affect all Gαq-mediated contractions more uniformly. Although iNOS induction is a hallmark of LPS action and contributes significantly to vasodilation our use of endothelium-denuded rings and focus on specific Gαq pathways suggests additional, direct effects on VSM signaling are operative. Some studies suggest iNOS itself can impair contraction partly via sGC activation but also impair relaxation. We propose that the observed differential effects may be related, at least in part, to the upregulation of RGS16. Our molecular data clearly show a significant increase in both RGS16 mRNA and protein levels in aortic VSM following LPS treatment. This is consistent with findings in other cell types where RGS16 is known to be an inflammation-inducible gene. RGS16 functions as a GAP primarily for Gαq/11 and Gαi/o subunits. By accelerating GTP hydrolysis on Gαq, upregulated RGS16 would be expected to attenuate signaling downstream of Gαq-coupled receptors. This provides a plausible mechanism for the observed hyporesponsiveness to phenylephrine.

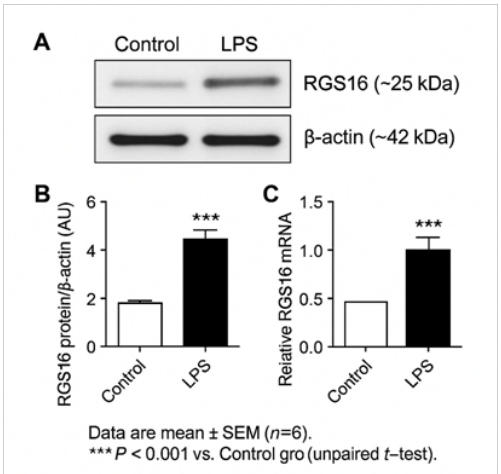

Figure 2: Effect of LPS on RGS16 expression in rat aorta.

Several possibilities exist. Firstly, RGS proteins can exhibit "receptor specificity" even when targeting the same Gα subunit. The efficiency of RGS-mediated GAP activity can be influenced by the specific receptor activated and the conformation it induces in the Gα subunit. It is conceivable that RGS16 is more effective at attenuating α1-AR-Gαq signaling compared to AT1R-Gαq or ETAR-Gαq signaling. Secondly, receptor signaling pathways are complex and involve scaffolding proteins and microdomain localization (e.g., caveolae) which might differentially regulate receptor access to RGS proteins. LPS might alter these organizational structures in a way that selectively shields certain receptors (like AT1R) from the inhibitory action of upregulated RGS16, while exposing others (like α1-AR). Thirdly, the enhanced sensitivity to Ang II could involve compensatory mechanisms or distinct signaling properties of the AT1R. For instance, AT1Rs can activate multiple signaling pathways beyond canonical Gαq/PLCβ, including pathways involving Gα12/13 or β-arrestin recruitment, which might be less sensitive to RGS16 or even upregulated by LPS. The relative resistance of ET-1 responses might be due to the sheer potency of ET-1 signaling or activation of redundant pathways (e.g., coupling to Gα12/13 alongside Gαq) that overcome moderate increases in RGS activity.

The induction of RGS16 by LPS in VSM represents a novel potential mechanism contributing to the complex vascular phenotype of sepsis. While often viewed purely as negative regulators, the differential impact of RGS proteins suggested here implies a more nuanced role. By selectively dampening certain vasoconstrictor pathways (α1-AR) while potentially sparing or even enhancing others (AT1R), RGS16 upregulation could contribute to the unpredictable and heterogeneous vascular responses seen clinically. This might explain why vasopressor combinations are sometimes required in septic shock management.This study has limitations inherent in its hypothetical nature and ex vivo design. The 4-hour incubation period represents an acute LPS challenge, and chronic exposure in vivo likely involves more complex interactions, including systemic cytokine release and infiltration of immune cells, which could further modify RGS expression and vascular function. While we focused on RGS16 due to its known Gαq selectivity and inducibility, LPS likely affects the expression and function of other RGS proteins (e.g., RGS2, RGS4, RGS5, also implicated in cardiovascular regulation) and other signaling modulators. Future studies would be needed to confirm these findings in vivo, to directly assess RGS16 GAP activity in LPS-treated VSM, and to use genetic tools (e.g., RGS16 knockout or siRNA knockdown) to definitively establish a causal link between RGS16 upregulation and the observed differential vascular responses. Investigating the potential interplay between RGS16 and NO/iNOS pathways would also be crucial, as NO itself can S-nitrosylate RGS proteins, potentially altering their activity.

Conclusion

LPS exposure induces a complex and differential modulation of vasoconstrictor responses in rat aortic smooth muscle. While responses to phenylephrine are significantly attenuated, sensitivity to angiotensin II is paradoxically enhanced, and endothelin-1 responses are relatively preserved. These functional changes correlate with a significant upregulation of RGS16 mRNA and protein levels in LPS-treated vascular tissue. We propose that the inducible expression of RGS16 by LPS contributes to this differential phenotype, potentially by selectively attenuating α1-adrenergic signaling more effectively than AT1R or ETAR signaling. These findings highlight RGS16 as a potential key player in the intricate signaling dysregulation underlying vascular dysfunction in sepsis and suggest that targeting specific RGS proteins could be a novel therapeutic avenue, although further investigation is required to substantiate this role in vivo and delineate the precise molecular mechanisms.

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