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E. coli lipopolysaccharide attenuates adenosine A₁ receptor-mediated increase in plasma exudation from the hamster cheek pouch

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Abstract

Objective and design—To determine whether exposure to *E. coli* lipopolysaccharide (LPS) modulates adenosine A₁ receptor-induced increase in plasma exudation from the intact hamster cheek pouch microcirculation.

Methods and results—Using intravital microscopy, we found that suffusion of *R*(-)-*N*⁶-(2-phenylisopropyl)-adenosine (*R*(-)-PIA) (1.0 and 10.0 nM), a selective adenosine A₁ receptor agonist, onto the intact cheek pouch elicited significant, concentration-dependent leaky site formation and increase in clearance of fluorescein thioisocyanate-dextran (mol mass, 70 kDa) from post-capillary venules ($p < 0.05$). These responses were significantly attenuated by pre-treatment of hamsters with LPS ($p < 0.05$). By contrast, LPS had no significant effects on CGS-21680-, a selective adenosine A_{2A} receptor agonist, bradykinin- and substance P-induced increases in plasma exudation from the cheek pouch.

Conclusion—These data indicate that LPS attenuates adenosine A₁ receptor-induced increase in plasma exudation in vivo in a specific fashion. We suggest that this phenomenon represents an endogenous anti-inflammatory cue to avoid excessive inflammation during Gram-negative bacterial infections.

Keywords

Infection; Inflammation; Post-capillary venules; Bradykinin; Substance P

Introduction

It is well established that exposure to lipopolysaccharide (LPS), a constituent of Gram-negative bacterial cell wall, increases plasma exudation from post-capillary venules in the peripheral microcirculation, a hallmark of the host inflammatory response to injury, leading to organ dysfunction [1]. To this end, adenosine is a potent mediator elaborated and released

in injured and inflamed tissues [2–5]. However, the role of adenosine in modulating plasma exudation during Gram-negative bacterial infections is uncertain [4, 6–8].

For example, Neely et al. [7] showed that adenosine A₁ receptor antagonists attenuate LPS-induced acute lung injury in cats. They proposed that these drugs may be efficacious in treating patients with adult respiratory distress syndrome elicited by Gram-negative septicemia. In contrast, Heller et al. [4] showed that adenosine A₁ receptor agonists attenuate LPS-induced increase in plasma exudation in rabbit lungs. Similar observations were reported by Schrier et al. [8] in carrageenan-induced pleural effusion in rats. Previous studies from our laboratory showed that adenosine increases plasma exudation from post-capillary venules in the intact golden Syrian hamster cheek pouch microcirculation, and that this response was mediated by adenosine A₁ receptors [6]. These apparent discrepant results on the role of adenosine A₁ receptors in modulating LPS-induced increase in plasma exudation from the peripheral microcirculation may be related, in part, to different animal models and microvascular beds used in these studies [4, 6–8].

Accordingly, the purpose of this study was to determine whether exposure to LPS modulates adenosine A₁ receptor-induced increase in plasma exudation from post-capillary venules in the intact hamster cheek pouch microcirculation and, if so, whether these effects are specific.

Materials and methods

General methods

Preparation of animals—Adult male golden Syrian hamsters (120–140 g body weight) were used in these studies as previously described in our laboratory and by other investigators [1, 6, 9–14]. Each animal was anesthetized with pentobarbital sodium (6 mg/100 g body weight i.p.). A tracheostomy was performed to facilitate spontaneous breathing. The left femoral vein was cannulated to inject the intravascular tracer, fluorescein isothiocyanate-labeled dextran (FITC-dextran; mol mass, 70 kDa; 40 mg/100 g body wt dissolved in 1.0 ml normal saline and administered over 1 min) and supplemental anesthesia (2–4 mg/100 g body weight/h). The left femoral artery was cannulated to obtain arterial blood samples and to monitor systemic arterial pressure and heart rate during the experiment. Body temperature was kept constant (37–38°C) during the experiment using a heating pad.

To visualize the microcirculation of the cheek pouch, we used a method previously described in our laboratory and by other investigators [1, 6, 9–14]. Briefly, the left cheek pouch was spread gently over a small plastic base plate and an incision was made in the skin to expose the cheek pouch membrane. The avascular connective tissue layer was carefully removed and a plastic chamber was positioned over the base plate and secured in place by suturing the skin around the upper chamber. This chamber contained the suffusion fluid. This arrangement forms a triple-layered complex: the base plate, the upper chamber and the cheek pouch membrane exposed between both plates. The hamster was then transferred to a heated microscope stage. The chamber was connected to a reservoir containing warmed (37–38°C) bicarbonate buffer (composition, in mM: NaCl, 131.9; KCl, 2.95; CaCl₂, 1.48; MgCl₂, 0.76; NaHCO₃, 11.87), which allowed continuous suffusion of the cheek pouch. The buffer was bubbled continuously with 95% O₂–5% CO₂ (pH = 7.4). The chamber was also connected via a three-way valve to an infusion pump (Sage Instruments, Cambridge, MA, USA) that allowed for constant administration of drugs into the suffusate.

Determination of clearance of macromolecules—The cheek pouch microcirculation was visualized with an Olympus microscope (Olympus America Inc, Melville, NY, USA)

coupled to a 100-W mercury light source at a magnification of 40 $\times$. Fluorescence microscopy was accomplished with the aid of filters that matched the spectral characteristics of FITC-dextran [1, 6, 9–14]. Macromolecular leakage was determined by extravasation of FITC-dextran, which appeared as fluorescent “spots” or leaky sites around post-capillary venules. The number of leaky sites was determined by counting three random microscopic fields every minute for the first 7 min and then at 5-min intervals for 30–60 min after each intervention (see below). The total number of leaky sites was averaged and expressed as the number of leaky sites per 0.11 cm² of cheek pouch, which corresponds to an area of one microscopic field.

In experiments in which clearance of FITC-dextran was calculated, the suffusate was collected at 5-min intervals throughout the experiment by a fraction collector (Microfractionator, Gilson Medical Electronics, Middleton, WI, USA). Samples were collected in glass tube tests and the concentration of FITC-dextran was determined in each tube. Arterial blood samples were collected in heparinized capillary tubes (70- μ l volume; Scientific Products, McGaw Park, IL, USA) beginning 5 min before and 5, 30, 60, 120, 180 and 240 min after intravenous injection of FITC-dextran. The concentration of FITC-dextran was determined in all plasma samples.

To quantitate the concentration of FITC-dextran in the plasma and suffusate, a standard curve for FITC-dextran concentrations versus percent emission was generated on a spectrophotofluorometer (Perkin-Elmer, Norwalk, CT, USA). The standard was FITC-dextran prepared on a weight per volume basis. With bicarbonate buffer as background, a standard curve was generated for each experiment and each curve was subjected to linear regression analysis. The percent emission for unknown samples (plasma and suffusate) was determined by the spectrophotofluorometer and the concentration of FITC-dextran was then calculated from the standard curve. In preliminary experiments, minimal fluorescence signal (<2% above background) was detected when drugs were added to the buffer and when plasma and suffusate samples were examined before adding FITC-dextran. Clearance of FITC-dextran was determined by calculating the ratio of suffusate (ng/ml) to plasma (mg/ml) concentration of FITC-dextran and multiplying this ratio by the suffusate flow rate (2 ml/min).

Experimental protocols

Effects of LPS on adenosine receptor agonist-induced increase in macromolecular efflux—The purpose of these studies was to determine whether exposure to LPS modulates adenosine receptor A₁ and A₂ agonists-induced increase in macromolecular efflux from intact hamster cheek pouch microcirculation. After suffusing buffer for 30 min (equilibration period), FITC-dextran was injected intravenously and the number of leaky sites and clearance of FITC-dextran were determined for 60 min. Then, increasing concentrations of *R*(-)-*N*⁶-(2-phenylisopropyl)-adenosine (*R*(-)-PIA) (1.0 and 10.0 nM), a selective adenosine A₁ receptor agonist [21], or CGS-21680 (1.0 and 5.0 μ M), a selective adenosine A_{2A} receptor agonist [6], were suffused onto the cheek pouch in a non-systematic fashion [6]. Each concentration was suffused for 5 min. The number of leaky sites was determined before and every minute for 7 min and at 5 min intervals for 45 min thereafter. Clearance of FITC-dextran was determined before and every 5 min thereafter for 45 min. The time interval between subsequent suffusions of each agonist was at least 45 min [6]. In another series of experiments, *E. coli* LPS (77 μ g/kg, i.p.) was administered 48 h before each experiment and suffusion of *R*(-)-PIA and CGS-21680 onto the cheek pouch was performed as outlined above. The dose of *E. coli* LPS used in these studies has been shown to exert reproducible metabolic effects in hamsters [4, 15, 16].

In preliminary studies, we determined that repeated suffusions of *R*(-)-PIA (1.0 and 10.0 nM) or CGS-21680 (1.0 and 5.0 μ M) onto the cheek pouch were associated with reproducible results. In addition, 48 h after i.p. injection of LPS suffusion of saline (vehicle) for the entire duration of the experiment was not associated with visible leaky site formation or significant increase in clearance of FITC-dextran. The concentrations of *R*(-)-PIA and CGS-21680 used in these experiments was based on a previous study in our laboratory [6].

Effects of LPS on bradykinin- and substance P-induced increases in macromolecular efflux

—The purpose of these studies was to determine whether the effects of LPS on *R*(-)-PIA-induced responses are specific by determining its effects on bradykinin- and substance P-induced increases in macromolecular efflux from the intact hamster cheek pouch microcirculation. We and others have previously shown that bradykinin and substance P each increases plasma exudation from the intact hamster cheek pouch in receptor-specific fashion [9–14]. The experimental design was identical to that outlined above except that bradykinin or substance P (each, 0.5 μ M) was suffused for 5 min onto intact cheek pouches of naïve hamsters and hamsters treated with LPS (77 μ g/kg, i.p.) 48 h before the experiment. In preliminary studies, we determined that repeated suffusions of bradykinin or substance P (each, 0.5 μ M) were associated with reproducible results. The concentrations of bradykinin and substance P used in these experiments were based on previous studies in our laboratory [10, 11].

All animal experiments were performed in accordance with procedures approved by the University of Illinois at Chicago Animal Care Committee. Animals were cared for in accordance with the principles of the “Guide for the Care and Use of Experimental Animals”.

Drugs and chemicals

Fluorescein isothiocyanate-labeled dextran, *R*(-)-PIA, CGS-21680, bradykinin and substance P were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). *E. coli* LPS 55:B5 was obtained from Difco Laboratories (Difco, MI, USA). All drugs and chemicals were prepared and diluted in saline to the desired concentrations on the day of experiment.

Data and statistical analyses

Data are expressed as means $\pm$ SEM. Because the number of leaky sites returned to baseline (nil) between successive applications of test compounds, all vehicle (saline) control data are expressed as a single value for each experimental condition. Statistical analysis was performed on actual values using repeated-measures analysis of variance with Neuman–Keuls multiple-range post hoc test to detect values that were different from control values. A $p < 0.05$ was considered statistically significant; n is given as the number of experiments, with each experiment representing a separate animal.

Results

Systemic arterial pressure and heart rate did not change significantly throughout the duration of experiments ($n = 38$ animals; $p > 0.5$).

Effects of LPS on adenosine receptor agonist-induced increase in macromolecular efflux

Suffusion of *R*(-)-PIA (1.0 and 10.0 nM), a selective adenosine A_1 receptor agonist, and CGS-21680 (1.0 and 5.0 μ M), a selective adenosine A_{2A} receptor agonist, elicited significant concentration-dependent increase in leaky site formation and clearance of FITC-dextran from cheek pouch post-capillary venules (Figs. 1, 2; each group, $n = 4$ animals; $p < 0.05$). Effects of *R*(-)-PIA on macromolecular efflux were observed at nanomolar

concentrations whereas those of CGS-21680 at micromolar range indicating adenosine A₁ receptors mediate this response as previously reported from our laboratory [6]. Pre-treatment with LPS significantly attenuated R(-)-PIA-induced increase in macromolecular efflux from cheek pouch but had no significant effects on CGS-21680-induced responses (Fig. 1; each group, $n = 4$ animals; $p < 0.05$, and Fig. 2; each group, $n = 4$ animals; $p > 0.5$, respectively).

Effects of LPS on bradykinin- and substance P-induced increase in macromolecular efflux

Suffusion of bradykinin and substance P (each, 0.5 μ M) elicited significant increase in leaky site formation and clearance of FITC-dextran (Fig. 3; each group, $n = 4$ animals; $p < 0.05$). Pre-treatment with LPS had no significant effects on either bradykinin- or substance P-induced increases in macromolecular efflux from cheek pouch (Fig. 3; each group, $n = 4$ animals; $p > 0.5$, respectively).

Discussion

The new finding of this study is that prolonged systemic exposure to LPS as observed in patients with Gram-negative bacterial infections attenuates adenosine A₁-induced increase in plasma exudation from the intact hamster cheek pouch microcirculation. This response was specific because LPS had no significant effects on CGS-21680-, a selective adenosine A_{2A} receptor agonist, bradykinin- and substance P-induced increases in plasma exudation from the cheek pouch. Administration of LPS alone at the dose used in this study 48 h before exposure to agonists had no significant effects of plasma exudation. Taken together, these data suggest that prolonged systemic exposure to LPS is associated with selective hyporesponsiveness of adenosine A₁ receptors in the intact hamster cheek pouch microcirculation and a decrease in plasma exudation. We suggest that this phenomenon represents an endogenous anti-inflammatory cue to avoid excessive inflammation during Gram-negative bacterial infections.

The mechanism(s) underlying LPS modulation of adenosine A₁ receptor-induced increase in plasma exudation from the intact hamster cheek pouch microcirculation was not elucidated in this study. Conceivably, prolonged systemic exposure to LPS could downregulate expression of adenosine A₁ receptors and/or downstream intracellular signaling cascade(s) in cheek pouch microcirculation either directly or through elaboration of cytokines and chemokines [4, 6–8, 17–24]. To this end, Ruiz and her colleagues [18] and León et al. [19] showed that prolonged exposure to selective adenosine A₁ receptor agonists, such as R(-)-PIA, decreases significantly the number of high-affinity adenosine A₁ receptors in rat brain. Likewise, Rebola et al. [20] found a long-term decrease in the number of adenosine A₁ receptors in rat brain after induction of convulsions coupled with increased density of adenosine A_{2A} receptors. However, Murphree and her colleagues [24] showed that exposure of mouse peritoneal macrophages to LPS is associated with no apparent change in adenosine A₁ receptor mRNA while at the same time adenosine A_{2A} receptor transcript was increased. Moreover, prolonged stimulation of adenosine A_{2A} receptors in porcine coronary artery endothelial cells was not associated with loss of functional response suggesting this receptor subtype does not desensitize after prolonged stimulation [21].

To the best of our knowledge molecular biology tools to probe adenosine receptor subtype gene expression and protein are not available for golden Syrian hamsters. Nonetheless, Rubinstein et al. [6] showed that *N*^G-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, and indomethacin, a non-selective cyclooxygenase inhibitor, had no significant effects on R(-)-PIA-induced increase in macromolecular efflux from the hamster cheek pouch. These data indicate that adenosine A₁ receptor-induced responses in this microvascular bed are nitric oxide- and prostaglandin-independent. It is well established that both metabolic pathways are modulated by LPS [24, 26]. Clearly, additional studies are

indicated to unravel the mechanism(s) underlying LPS modulation of adenosine A₁ receptor responsiveness in the peripheral microcirculation of laboratory animals for which molecular biology tools to probe adenosine receptor subtype gene expression and protein are available. These studies will also determine the time course of the reduction in plasma exudation in response to R(-)-PIA 2–48 h after i.p. injection of LPS.

The hamster cheek pouch is an established animal model to study the effects of pro-inflammatory mediators, including LPS, adenosine and its receptor subtype agonists, bradykinin and substance P, on macromolecular efflux from post-capillary venules in situ and the mechanisms underlying these phenomenon [1, 6, 9–14, 25, 26]. In this model, solute efflux is determined by two reproducible parameters, leaky site formation and clearance of FITC-dextran, thereby providing quantitative appraisal of macromolecular transport across post-capillary venules in cheek pouch during experimental interventions. Importantly, successive suffusions of test compounds at appropriate time intervals are associated with reproducible formation of leaky sites and increases in clearance of FITC-dextran in absence of tachyphylaxis. Thus, changes in macromolecular efflux can be tested repeatedly in the same cheek pouch so that each animal serves as its own control. This, in turn, reduces the overall number of animals required to conduct the study and facilitates data analysis [1, 6, 9–14, 25, 26].

The effects of LPS on R(-)-PIA-induced increase in plasma exudation from the cheek pouch may have been mediated, in part, by changes in vasomotor tone and/or increase in venular driving pressure in the cheek pouch [1, 6, 9–14, 25–28]. However, this possibility seems unlikely because LPS had no significant effects on CGS-21680-, bradykinin- and substance P-induced responses.

In summary, we found that prolonged exposure to LPS selectively attenuates adenosine A₁ receptor-induced increase in plasma exudation from the intact hamster cheek pouch microcirculation in a specific fashion. We suggest that these effects limit adenosine-induced increase in plasma exudation during Gram-negative bacterial infection.

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Fig. 1. Effects of suffusion of *R*(-)-*N*⁶-(2-phenylisopropyl)-adenosine, a selective adenosine A₁ receptor agonist, on leaky site formation (*upper panel*) and clearance of FITC-dextran (*lower panel*) from intact hamster cheek pouch microcirculation in naïve animals (*closed bars*) and animals pre-treated with *E. coli* LPS (77 µg/kg, i.p.) 48 h before experiment (*open bars*). Data are means ± SEM. Each group, *n* = 4 animals; **p* < 0.05 in comparison to saline; †*p* < 0.05 in comparison to naïve hamsters

Fig. 2.

Effects of suffusion of CGS-21680, a selective adenosine A_{2A} receptor agonist, on leaky site formation (*upper panel*) and clearance of FITC-dextran (*lower panel*) from intact hamster cheek pouch microcirculation in naïve animals (*closed bars*) and animals pre-treated with *E. coli* LPS (77 μg/kg, i.p.) 48 h before experiment (*open bars*). Data are means ± SEM. Each group, *n* = 4 animals; **p* < 0.05 in comparison to saline

Fig. 3.

Effects of suffusion of bradykinin or substance P (each, 0.5 μM) on leaky site formation (*upper panel*) and clearance of FITC-dextran (*lower panel*) from intact hamster cheek pouch microcirculation in naïve hamsters and animals pre-treated *E. coli* LPS (77 μg/kg, i.p.) 48 h before experiment. Data are means ± SEM. Each group, $n = 4$ animals; * $p < 0.05$ in comparison to saline