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Protective effects of endothelin-A receptor antagonist BQ123 against LPS-induced oxidative stress in lungs

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Abstract:
The aim of this study was to assess whether endothelin-A receptor (ET_A-R) blocker, BQ123, influences lung edema, lipid peroxidation (TBARS), hydrogen peroxide (H_2O_2), TNF-α concentration or the glutathione redox system in the lung homogenates obtained from LPS-induced endotoxic shock rats. The study was performed on male Wistar rats (n = 6 per group) divided into groups: (1) saline, (2) LPS (15 mg/kg)-saline, (3) BQ123 (0.5 mg/kg)-LPS, (4) BQ123 (1 mg/kg)-LPS. The ET_A-R antagonist was injected intravenously 30 min before LPS administration. Five hours after saline or LPS administration, animals were sacrificed and lungs were isolated for indices of lung edema, oxidative stress and TNF-α concentration. Injection of LPS alone resulted in lung edema development and a marked increase in TNF-α ($p < 0.02$), TBARS ($p < 0.02$), and H_2O_2 ($p < 0.01$) concentrations as well as a depletion of total glutathione ($p < 0.01$). Administration of BQ123 (1 mg/kg), before LPS challenge, led to a significant reduction in TNF-α and H_2O_2 concentrations ($p < 0.05$) and elevation of both total glutathione and the GSH/GSSG ratio ($p < 0.05$). However, it did not prevent LPS-induced TBARS increase and lung edema formation. Interestingly, a lower dose of BQ123 was much more effective in decreasing H_2O_2, TBARS, as well as TNF-α levels ($p < 0.02$, $p < 0.05$, $p < 0.05$, respectively). That dose was also effective in prevention of lung edema development ($p < 0.01$). Taken together, the obtained results indicate that BQ123 is highly effective in decreasing LPS-induced oxidative stress in lungs. Moreover, the dose of 0.5 mg/kg of the antagonist showed to be more effective in decreasing free radical generation and lung edema in endotoxemic rats.

Key words:
BQ 123, oxidative stress, endotoxic shock, lung edema

Abbreviations: ET-1 – endothelin 1, ET_A-R – type A of endothelin receptor, ET_B-R – type B of endothelin receptor, GSH – reduced glutathione, GSSG – oxidized glutathione, GSH/GSSG ratio – reduced/oxidized glutathione ratio, GPx – glutathione peroxidase, H_2O_2 – hydrogen peroxide, LPS – lipopolysaccharide, MAPK – mitogen-activated protein kinases, NF-κB – nuclear factor kappa B, ROS – reactive oxygen species, TNF-α – tumor necrosis factor alpha

Introduction

Endothelin-1 (ET-1), a peptide of 21 amino acids, was shown to be one of the most potent vasoconstrictor compounds produced predominantly by endothelial cells [34]. In the lungs, ET-1 is generally synthesized

by smooth muscle cells but also by the epithelium, macrophages and pulmonary cells [7, 9, 24]. Various injurious stimuli including endotoxines, cytokines, and ROS, increase ET-1 releasing [7]. ET-1 action contributes to a long-lasting vasoconstriction of the smooth muscle cells which is mediated *via* activation of ET_A-R and ET_{B2}-R. These receptors, localized on pulmonary arteries, airway smooth muscles, and alveolar wall tissue capillaries, are coupled to Gq/11, G12/13 and Gi heterotrimeric G protein subunits, which bind to phospholipase C and RhoA-GTPase and eventually stimulate release of calcium from the endoplasmic reticulum [1, 8]. Acting simultaneously on ET_{B1}-R placed on endothelial cells, ET-1 stimulates nitric oxide, prostacyclin and endothelium derived relaxation factor (EDHF) release which leads to a short-lasting vasodilatation [10, 23]. The signaling of NO generation by ET_{B1}-R receptors *via* G-protein $\beta\gamma$ subunit dimer has been suggested as an effect of Akt phosphorylation of endothelial nitric oxide synthase (eNOS) [1].

ET-1 shows a variety of biological functions which include hemodynamic, cardiac and pulmonary changes [2, 25]. This peptide is also involved in the inflammatory process, overproduction of cytokines and free radicals. ET-1-induced bronchoconstriction, mucous secretion and cellular proliferation lead to severe lung disorders [7]. Moreover, ET-1 increases capillary permeability that is dependent on the presence of leukocytes and upregulation of mediators which enhance endothelial permeability e.g., vascular endothelial growth factor (VEGF) [18]. In animal models, the inflammatory process and overproduction of free radicals are frequently triggered by endotoxins (lipopolysaccharide, LPS). LPS binds to Toll-like receptors, activates NF-κB transcription factor, and increases pro-inflammatory mediators which, in turn, increase endothelial permeability [21] and pulmonary production of ET-1 [11, 31]. Numerous studies have indicated that ET-1 stimulates free radicals and cytokine generation *via* ET_A-R because the blockage of ET_A-R decreases ET-1-induced free radical generation, whereas ET_B-R inhibition has no significant effects [4, 6]. Recently, Chen et al. [5] have shown that BQ123, an ET_A-R blocker, inhibits pulmonary apoptosis and reduces the concentrations of inflammatory cytokines TNF-α and IL-1β as well as improves plasma antioxidant activity in rats with emphysema. In addition, ET-1 receptor blockers have been established as the first line option for patients with pulmonary arterial hypertension [22]. However, as far as the authors are aware, there have not been any studies dealing with ET_A-R blockers role in the oxidative stress and lung edema development accompanying pulmonary endotoxemia.

Therefore, we hypothesize that LPS induced oxidative stress is mediated by ET-1 and ET_A-R. In the present study, the effects of LPS on lung edema, free radical and cytokine generation as well as redox status of lung tissue were analyzed after pretreatment with different doses of the selective ET_A-R antagonist, BQ123. To the best of our knowledge it is for the first time that BQ123 inhibitory action on lung edema development and ROS and cytokine release in animal model of endotoxemia was examined.

Materials and Methods

Animals

Male Wistar rats (180–230 g) were kept under standard laboratory temperature (20 ± 2°C) and lighting (light from 6:00 to 18:00), with free access to lab chow and tap water, until used in the experiments. The experimental procedures followed the guidelines for the care and use of laboratory animals, and were approved by the Medical University of Lodz Ethics Committee No. 20/Ł418/2008.

Experimental protocol

Animals were divided into four groups (n = 6 per group). In group 1 (control group), rats received *iv* 0.6 ml of 0.9% NaCl and 30 min later again 0.6 ml of 0.9% NaCl. In group 2 (LPS-saline), rats received *iv* 0.6 ml of saline and 30 min later 0.6 ml of LPS (15 mg/kg). In groups 3 and 4 (BQ123-LPS), rats received *iv* 0.6 ml of BQ123 (0.5 mg/kg and 1 mg/kg, respectively) and 30 min later 0.6 ml of LPS (15 mg/kg). The animals were anesthetized by an intraperitoneal injection of urethane solution (60 mg per 100 g of body weight). When a sufficient level of anesthesia was achieved the skin and subcutaneous tissues on the neck were infiltrated with 2% polocaine hydrochloride solution (Polfa, Poland), a 2-cm-long polyethylene tube (2.00 mm O.D.) was inserted into the trachea. The right femoral artery was catheterized

and a polyurethane cannule was inserted (0.41 mm O.D.; 0.23 mm I.D.). All drugs were administrated directly into the femoral vein. Five hours after the last injection, rats were sacrificed. The thorax was opened and the lungs were harvested and rinsed with ice-cold saline to remove the excess of blood. The left lung (about 500 mg) was cut off, weighed and frozen in –75°C until used for measurements of oxidative stress parameters and TNF-α. The right lung (about 500 mg) was cut off, weighed and used immediately for the lung edema assay. There were massive hemorrhage foci found in the lungs of rats after BQ123 administration (post-experimental observations did not supported by histological studies).

Chemicals

Urethane, horseradish peroxidase (HRP), homovanillic acid (HVA), triethanolamine hydrochloride (TEA), 5-sulfosalicylic acid hydrate (5-SSA), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), β-NADPH (β-nicotinamide adenine dinucleotide phosphate), glutathione reductase (GR), 2-vinylpyridine, HEPES and enzyme inhibitors (phenylmethanesulfonyl fluoride and ovomucoid trypsin inhibitor from white egg), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were obtained from POCh (Gliwice, Poland) and were of analytical grade.

Lipopolysaccharide (Escherichia coli LPS 026:B6) and BQ123 were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in 0.9% NaCl just before the intravenous administration.

Assessment of lung edema

The removed lungs were immediately weighed to measure the wet weight and then placed in a drying oven at 80°C for 4 h, and again weighed (the dry weight). Finally, the lung wet-to-dry weight ratios were determined by dividing the wet weight by the dry weight and used as an indicator of lung edema.

Determination of lipid peroxidation

The lipid peroxidation product content in lung homogenates was assayed as TBARS, previously described by Yagi [33]. Briefly, 50 mg of frozen tissue was homogenized in 2 ml of 1.15% KCl using glass homogenizer. Next, 1 ml of homogenate was boiled for 15 min in a mixture containing 0.015% butylated hydroxytoluene, 0.375% thiobarbituric acid and 15% trichloroacetic acid. After cooling, 2.5 ml of butanol was added, vortexed and centrifuged at 3,000 rpm for 10 min. Thiobarbituric acid reactive substances (TBARS) were measured spectrofluorometrically using a Perkin Elmer Luminescence Spectrometer LS-50 (Norwalk, CT, USA). Excitation was set at 515 nm and emission was measured at 546 nm. Readings were converted into μM using the calibration curve obtained for 1,1,3,3-tetramethoxypropane (0.01–50 μM). Finally, the results were calculated for 50 mg of the lung tissue.

Determination of H_2O_2

Generation of H_2O_2 in lung homogenates was determined according to the Ruch et al. method [26]. Fifty mg of the frozen lung tissue fragments was homogenized with 2 ml of 1.15% KCl. Then, 10 μl aliquot of the resulting homogenate was divided between two Eppendorf tubes. In one tube, a mixture of phosphate buffered saline (PBS) (pH 7.0) and horseradish peroxidase (HRP) (1 U/ml) containing 400 μmol homovanillic acid (HVA) was added to assay HRP + HVA, while PBS and 1 U/ml HRP were added to the other tube to assay HRP. Both tubes were simultaneously incubated for 60 min at 37°C. Subsequently, PBS and 0.1 M glicyne-NaOH buffer (pH 12.0) with 25 mM EDTA were added to each Eppendorf tube to stop the enzymatic reaction. Excitation was set at 312 nm and emission was measured at 420 nm (Perkin Elmer Luminescence Spectrometer, Beaconsfield, UK). Readings were converted into H_2O_2 concentration using the regression equation prepared from three series of calibration experiments with 10 increasing H_2O_2 concentrations (range 10–1000 μM).

Determination of total, oxidized and reduced glutathione levels

Total glutathione (tGSH), reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in lung homogenates. Briefly, 50 mg of the frozen tissue was homogenized in cold 5% 5-SSA and centrifuged (10,000 × g, 10 min, 4°C). The collected supernatant was frozen at –80°C. The total GSH determination was performed in a 1 ml cuvette containing 0.7 ml of 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.1 ml of 0.6 mM DTNB, 0.150 ml of H_2O and 50 μl of unfrosted supernatant. The cuvette with the mixture was incubated for 5 min at 37°C and then

supplemented with 0.6 U of GR. The reaction kinetics was followed spectrophotometrically (Pharmacia LKB-Ultrospect III) at 412 nm for 5 min by monitoring the increase in absorbance.

The GSSG concentration was determined in the unfrozen supernatant in the same way after the optimization of pH to 6–7 with 1 M TEA and derivatization of endogenous GSH with 2-vinylpyridine (v:v). The reduced supernatant GSH level was calculated as the difference between total GSH and GSSG. The increments in absorbance at 412 nm were converted to GSH and GSSG concentrations using a standard curve (3.2–500 μM GSH for total GSH and 0.975–62 μM for GSSG). The results were expressed in μM.

Lung TNF-α assay

Lung homogenates were prepared by grinding 50 mg of frozen tissue in HEPES buffer (20 mM HEPES (pH 7.6), 1.5 mM EDTA, 0.5 mM benzamidine and enzyme inhibitors). After centrifuging (18,000 rpm, 4°C, 20 min), the supernatant was diluted and the pulmonary levels of TNF-α were quantified according to the manufacturer's instructions using specific ELISA kits for rats (Quantikine TNF-α, R&D Systems, USA). The absorption was read at 450 nm (ELx800 Bio-Tech Instruments reader). Values were expressed as pg/ml.

Statistical analysis

The data are presented as the mean ± SEM. The statistical analysis was performed using ANOVA followed by the Duncan's multiple range test as *post-hoc*. A p value lower than 0.05 was considered significant.

Results

Assessment of lung edema

LPS challenged caused an increase in wet-to-dry (W/D) ratio compared with the control ($p < 0.05$). Moreover, a lower dose of BQ123 (0.5 mg/kg) significantly ($p < 0.01$) reduced the W/D ratio, while a higher dose of the blocker was ineffective in reducing LPS-induced lung edema ($p > 0.05$) (Tab. 1).

Changes in lung homogenate oxidative injury

Changes in oxidative damage markers are presented in Table 1. Injection of LPS alone resulted in elevated TBARS ($p < 0.02$) and H_2O_2 ($p < 0.01$) concentrations as compared with the control group. The administration of 1 mg/kg of BQ123 caused a significant decrease in H_2O_2 production ($p < 0.05$) but only slightly decreased the lipid peroxidation level ($p > 0.05$) compared with the LPS group. Interestingly, a dose of 0.5 mg/kg of the ET_A-R blocker led to about a 50% decrease in the oxidative damage of lipids ($p < 0.05$) and it effectively decreased hydrogen peroxide generation ($p < 0.02$) in comparison with the LPS group.

As glutathione is one of the most powerful cellular antioxidants, the levels of tGSH, (GSSG, GSH and GSH/GSSG ratio) were determined in the present study. The status of glutathione metabolism was markedly increased after BQ123 administration both at the lower ($p < 0.05$) and the higher dose ($p < 0.05$) (Tab. 1). There was about 2.5-fold increase in tGSH and GSH in the lungs of rats that received a dose of 1 mg/kg of the blocker. The dose of 0.5 mg/kg was found 50% weaker in GSH reconstruction; however,

Tab. 1. Oxidative damage markers in lung homogenates in the control and after LPS (15 mg/kg), BQ123 (0.5 mg/kg), and BQ123 (1 mg/kg) administration (n = 6 per group; the mean ± SEM)

Oxidative damage markers \ Group	Control (n = 6)	LPS (15 mg/kg) + saline (n = 6)	BQ 123 (0.5 mg/kg) + LPS (15 mg/kg) (n = 6)	BQ 123 (1 mg/kg) + LPS (15 mg/kg) (n = 6)
TBARS (μM)	48.45 ± 2.4	112.88 ± 12.4##	57.35 ± 3.7*	91.02 ± 5.3
H_2O_2 (μM)	33.36 ± 3.9	140.92 ± 18.0###	45.24 ± 11.8**	77.18 ± 14.9*
GSH/GSSG ratio	3.4 ± 1	2.66 ± 1.2	18.45 ± 7.4*	33.05 ± 6.7*
Wet-to dry ratio	4.8 ± 1.4	17.3 ± 3.6#	10 ± 1.2***	16.3 ± 0.3

* $p < 0.05$; ** $p < 0.02$; *** $p < 0.01$ *vs.* LPS group. # $p < 0.05$; ## $p < 0.02$; ### $p < 0.01$ *vs.* control

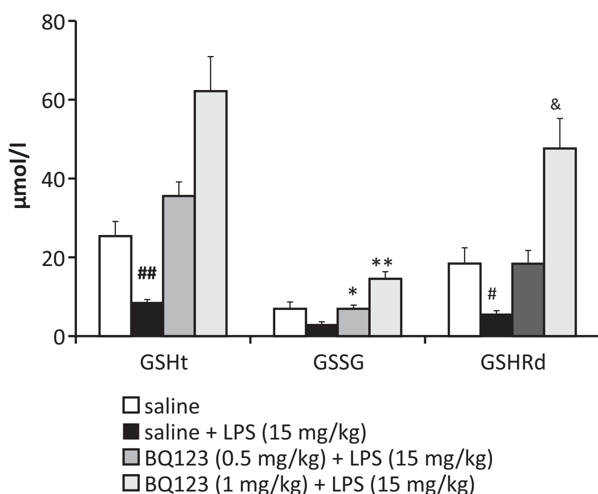

Fig. 1. Total, oxidized and reduced glutathione levels in lung homogenates in the control, and after LPS (15 mg/kg), BQ123 (0.5 mg/kg), and BQ123 (1 mg/kg) administration (n = 6 per group; the mean ± SEM). * $p < 0.05$, ** $p < 0.01$ *vs.* LPS group; $^{\&}$ $p < 0.02$ *vs.* BQ123 (0.5 mg/kg); $^{\#}$ $p < 0.02$, $^{\#\#}$ $p < 0.01$, *vs.* control. GSHt – total glutathione; GSSG – oxidized glutathione; GSHRd – reduced glutathione

it significantly averted the LPS-induced GSH depletion ($p < 0.02$) (Fig. 1).

TNF-α concentration

To further confirm the effects of BQ123 on the LPS-induced lung inflammation, TNF-α concentration was determined. In LPS treated rats TNF-α level was over 5-fold higher than in the control group ($p < 0.02$). BQ123 significantly ($p < 0.05$) but not in a dose dependent manner, inhibited TNF-α formation in endotoxemic rats (Fig. 2).

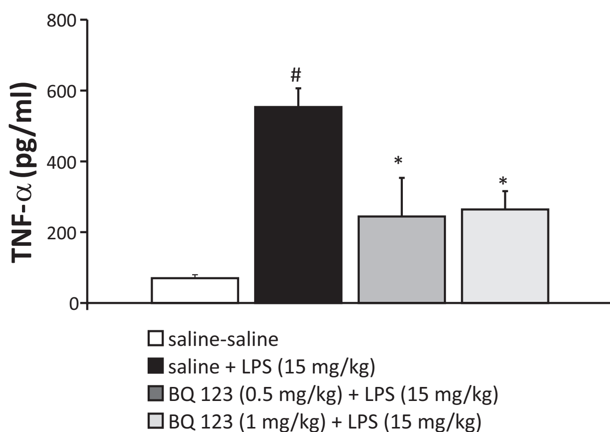

Fig. 2. TNF-α concentration in lung homogenates in the control and after LPS (15 mg/kg), BQ123 (0.5 mg/kg) and BQ123 (1 mg/kg) administration (n = 6 per group; the mean ± SEM). * $p < 0.05$ *vs.* LPS group; $^{\#}$ $p < 0.02$ *vs.* control

Discussion

In our study we showed that administration of LPS caused an increase in lipid peroxidation products and hydrogen peroxide concentration as well as a decrease in the GSH/GSSG ratio in lung homogenates. That indicates that LPS led to ROS overproduction and the development of oxidative stress which was reflected, in our study, by severe lung edema. Our results are in accordance with those obtained by other authors who proved that intravenous administration or inhalation of LPS lead to generalized inflammation, tissue injury and septic shock [21, 29, 32]. In a previous study we also demonstrated that LPS increased the TBARS level in the lung tissue [12] and in the bronchoalveolar lavage fluid (BALF) [13]. This increase may result from ROS overproduction and depletion of superoxide dismutase (SOD), catalase (CAT), as well as from decreased glutathione level [28]. Moreover, the activation of NF-κB and MAPK pathways in LPS-injured lung cells stimulate the release of cytokines and nitric radicals, which, similarly to ROS, impair the pulmonary vascular function and stimulate ET-1 production [16]. ET-1, in turn, causes massive vasoconstriction *via* ET_A-R and stimulates generation of free radicals [11, 31]. Bhavsar et al. [3] demonstrated markedly increased concentrations of lung neutrophils and other pro-inflammatory cells after ET-1 injection to endotoxemic hamsters, and those diminished after applying ET_A-R blocker.

Most of *in vivo* studies recommend 1 mg/kg of BQ123 as efficient in decreasing production of free radicals and pro-inflammatory cytokines in lungs [19, 30]. However, our study has shown that only the lower dose of BQ123 (0.5 mg/kg) decreased both lung edema and ROS generation, while the higher dose of BQ123 (1 mg/kg), proved H_2O_2 production but was ineffective in preventing lipid peroxidation and lung edema development. A decease in ROS production after the lower dose of the blocker was reflected by a decrease in lipid peroxidation and H_2O_2 concentration in endotoxemic rats. Therefore, the results of our study might suggest that a lower dose of the blocker (0.5 mg/kg) is potent and effective in de-

creasing oxidative stress, cytokine overproduction as well as lung edema development during endotoxemia. However, there has been no study available in the literature, where BQ123 would be used in a dose lower than 1 mg/kg during lung endotoxemia. Therefore, the mechanism of the protective role of BQ123 used at a dose of 0.5 mg/kg in our study seems unclear and needs to be explained. In addition, ET_A-R or $ET_A/_B$-R antagonists but not ET_B-R antagonists have been reported to play an important role in lung injuries due to generalized inflammation [15]. BQ123 was shown to reduce airway hyperactivity in asthma *via* decreasing eosinophil infiltration [19]. The latest study also demonstrated that dual $ET_A/_B$-R antagonist, tezosentan, attenuated lung injury induced by aortic ischemia/reperfusion in rats, which was linked with an increase in catalase and myeloperoxidase activation [17].

Tissue GSH plays a major role in the antioxidant defense by detoxifying ROS directly or in a GPx-catalized mechanism. In the lungs, GSH is depleted upon its conversion to the oxidized form GSSG (disulfide) during the removal of hydrogen peroxide. Thus, LPS-induced lung inflammation markedly decreased the GSH level and intensified ROS mediated lung injury. In our study, BQ123 not only prevented LPS-injury but also increased the total GSH level. Moreover, these findings are inversely related to the oxidative stress level during which ROS are produced and lungs are damaged.

Cytokines and chemokines play a key role in the activation of macrophages and neutrophils during the inflammatory process. In our study, LPS markedly increased TNF-α concentration in the lung tissue which is in agreement with the results obtained by other authors [20]. Overproduction of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 was observed in patients with lung injury [14] and in mice after LPS-induced acute lung injury [35]. Sampaio et al. [27] reported that intrathoracic administration of BQ123 sufficiently decreased TNF-α and IL-6 levels in septic mice. Similar results were obtained by Kassuya et al. [15] who found that injection of BQ123 decreased lung edema probably through decreasing levels of inflammatory mediators. In our study, a dose of 0.5 mg/kg of BQ123 was as effective in decreasing TNF-α concentration in endotoxemic rat lungs as a dose of 1 mg/kg.

In conclusion, our study showed that BQ123 at the dose of 0.5 mg/kg prevented LPS-induced lung injury by decreasing the oxidative stress parameters and lung edema development more effectively than a dose of 1 mg/kg.

Limitations

We hypothesize that stronger effects of 0.5 mg/kg of BQ123 (the lower dose) on lung injury may be due to: firstly, weaker stimulation of lung inflammatory cell activation and hydrogen peroxide secretion during respiratory burst, and secondly to the limited duration time of our experiment (endotoxemia persisted for about 3 h before the rats were sacrificed). This could have disabled the higher dose to act efficiently on pro-inflammatory cell stimulation and in turn, on free radical overproduction as it was shown in the previously cited reports, where the dose of 1 mg/kg of BQ123 decreased ROS production [5, 30]. However, those authors used BQ123 only in a dose of 1 mg/kg and did not administer any lower doses.

Therefore, in our study based on lung edema occurrence and TNF-α concentration assay, we assumed that a dose of 0.5 mg/kg of BQ123 was more potent in decreasing oxidative stress which led to lung injury under endotoxemia. However, there has been no study available in the literature, where BQ123 would be used at a dose lower than 1 mg/kg during lung endotoxemia. Therefore, the mechanism of the protective role of BQ123 used at a dose of 0.5 mg/kg in our study seems unclear and needs to be further explained.

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