The influence of ETA and ETB receptor blockers on LPS-induced oxidative stress and NF-κB signaling pathway in heart

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Abstract. The aim of this study was to assess whether an endothelin-A receptor (ETA-R) blocker, BQ123, or an endothelin-B (ETB-R) receptor blocker, BQ788, influences nuclear factor kappa beta (NF-κB) pathway, free radical generation, tumor necrosis factor-alpha (TNF-α) concentration, and glutathione redox system in hearts obtained from lipopolysaccharide (LPS)-induced endotoxic rats. The study was performed on rats divided into groups: 1) saline, 2) saline + LPS (15 mg/kg), 3) BQ123 (1 mg/kg b.w.) + LPS, 4) BQ123 (0.5 mg/kg b.w.) + LPS, 5) BQ788 (3 mg/kg b.w.) + LPS. The ETA-R and ETB-R antagonists were injected i.v. 30 min before LPS administration. In rats, BQ123 caused a significant decrease in TBARS ($p < 0.05$) but not in $H_2O_2$ concentration. It also decreased tissue protein level and improved tissue redox status ($p < 0.01$). Only a dose of 1 mg/kg decreased TNF-α concentration ($p < 0.05$). BQ788 lowered TBARS, $H_2O_2$ and protein concentration ($p < 0.05; p < 0.02; p < 0.001$, respectively), however, it did not affect TNF-α concentration. Neither ETA-R nor ETB-R blockers influenced LPS-induced increase in p65 subunit level and activation of NF-κB pathway. Our results demonstrated that ETA-R blockage is more effective in inhibiting free radical generation and improving heart antioxidant properties than ETB-R blockage under oxidative stress. NF-κB pathway is not incorporated in ETA-R and ETB-R influence on ROS production.

Key words: Endothelin-1 receptors — Oxidative stress — NF-κB — Heart

Abbreviations: ET-1, endothelin 1; ETA-R, type A of endothelin receptor; ETB-R, type B of endothelin receptor; GSH, reduced glutathione; GSSG, oxidized glutathione; GSH/GSSG ratio, reduced/oxidized glutathione ratio; GSH, total glutathione; GPx, glutathione peroxidase; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; TNF-α, tumor necrosis factor-alpha.

Introduction

Oxidative stress is a consequence of excessive production of reactive oxygen species (ROS) or insufficient level of antioxidants. ROS are present in relative excess in various pathological states, including endotoxemia, myocardial ischemia, and congestive heart failure. It is well established that during this condition upregulation of endothelin-1 (ET-1) gene expression and secretion is observed (Konrad et al. 2007). ET-1 is a 21-amino acid signaling molecule and growth factor produced and released by the vascular endothelium and cardiomyocytes in the heart. ET-1 binds to two distinct receptors ETA (ETA-R) and ETB (ETB-R) (Yamamoto et al. 2005). In the heart, both ETA-Rs and ETB-Rs are localized in the myocardium, endocardium, conducting system, and coronary vessels (Molenaar et al. 1993; Awane-Igata et al. 1997). ETA-Rs and ETB2-Rs are responsible for constriction of vessels and myocytes while activation of ETB1-Rs result in nitric oxide (NO) and prostacyclin release that leads to short-lasting dilatation. However, the role of each subtype in heart oxidative stress during endotoxemia has not been completely clarified.

It has been indicated that augmented ROS production upregulates ET-1 gene expression and stimulates release of ET-1 in vessels (Kähler et al. 2000). ROS generation is mostly a consequence of ETA-Rs stimulation but ETB-Rs...
play an additive role via stimulation of NADPH oxidase (Dong et al. 2005). Among the ET-1 receptor selective blockers BQ123, ETA-R blocker is frequently used in preclinical and clinical research of ET-1. It has been demonstrated to decrease mortality and improve hemodynamic parameters in experimental models of heart failure (Ozdemir et al. 2006). Recent reports have indicated inhibitory effect of BQ123 on lipid peroxidation and, therefore, on myocardial injury depletion (Goyal et al. 2010). Conversely, there are some experimental studies where specific ETA-R blockers or dual ETA/ETB-R blockers do not show any cardioprotection (Krause et al. 1994).

The preproET-1 promoter region has been shown experimentally to possess binding sites for nuclear factor kappa B (NF-κB), and NF-κB activation is related to ET-1 overexpression (Wort et al. 2009). Furthermore, ROS activate the endothelial cell to release more ET-1. The interaction among the inflammatory factors supports the concept that inflammatory factors may be modulated by ETA-Rs or ETB-Rs. Xia et al. (2006) showed that ETA-R blockage suppressed the upregulated mRNA level of NF-κB and TNF-α in fibrillated ventricles. Moreover, Wang et al. (2010) indicated that ET-1 activated NF-κB signaling pathway via ETB-Rs and activation of c-Src-dependent PI3K/Akt and p42/p44 MAPK in the central nervous system. However, the participation of ET-1 receptors and NF-κB signaling pathway under oxidative stress in the heart remains unclear.

The aim of the present study was to test the interaction between the ET-1 receptor blockade, NF-κB activation and the free radical and cytokine production during lipopolysaccharide (LPS)-induced endotoxemia in the heart. Specifically, the effect of BQ123, selective ETA-R antagonist, and BQ788, selective ETB-R antagonist, was investigated on the free radical generation, tissue redox status, TNF-α production and the level of p65 protein of NF-κB signaling pathway.

Material and Methods

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 were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and BQ788 were dissolved in 0.9% NaCl just before the intravenous administration.

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/L418/2008.

Experimental protocol

Animals were divided into five groups (n = 6 per group).

- Group 1 (saline): rats received 0.6 ml of 0.9% NaCl and 30 min later again 0.6 ml of 0.9% NaCl
- Group 2: rats received 0.6 ml of saline and 30 min later 0.6 ml of LPS (15 mg/kg)
- Group 3 and 4: rats received 0.6 ml of BQ123 (0.5 mg/kg or 1 mg/kg, respectively) and 30 min later 0.6 ml of LPS (15 mg/kg)
- Group 5: rats received 0.6 ml of BQ788 (3 mg/kg) and 30 min later 0.6 ml of LPS (15 mg/kg).

Animals were anaesthetized with 10% urethane (2 ml/100 g b.w.). Trachea and femoral vein were dissected. All drugs were administered directly into the femoral vein. Five hours after saline or LPS administration, animals were sacrificed under anesthesia and the hearts were isolated for further measurements.

Determination of lipid peroxidation

The lipid peroxidation products content in heart homogenates was assayed as thiobarbituric acid reactive substances (TBARS), previously described by Yagi et al. (1986). TBARS were measured spectrophotometrically 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 range using the calibration curve obtained for tetramethoxypropane (0.01–50 µM). Finally, the results were calculated for 50 mg of the heart tissue.

Determination of hydrogen peroxide concentration

The generation of H$_2$O$_2$ in heart homogenates was determined according to the method of Ruch et al. (1983). 50 mg of the heart tissue fragments was homogenized with 2 ml of 1.15% potassium chloride and the H$_2$O$_2$ concentration was measured using HRP/HVA systems. Samples were incubated for 60 min at 37°C, enzymatic reaction was
stopped by adding 0.1 M of glycine-NaOH buffer (pH 12.0) with 25 mM of EDTA. The excitation was set at 312 nm and the emission was measured at 420 nm (Perkin Elmer luminescence spectrometer, Beaconsfield, UK). Readings were converted into H$_2$O$_2$ concentration using the regression equation prepared from three series of calibration experiments with 10 increasing H$_2$O$_2$ concentrations (range 10–1000 µM).

**Determination of protein level**

The protein content in the heart homogenates was measured with the spectrophotometric method described by Lowry et al. (1951). The values for absorbance at 750 nm (Pharmacia LKB Ultraspec III, UV/VISIBLE spectrophotometer, Beaconsfield, UK) were converted to the protein concentration with 2-vinylpyridine (v:v). The reduced GSH level in the supernatant was calculated as the difference between total GSH and GSSG. The increments in absorbance at 412 nm were converted into H$_2$O$_2$ concentration using the regression equation prepared from three series of calibration experiments with 10 increasing H$_2$O$_2$ concentrations (range 10–1000 µM).

**Tissue redox status measurement**

Total glutathione (tGSH), reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in heart homogenates. Briefly, hearts were homogenized in cold 5% 5-SSA and centrifuged (10,000 × g, 10 min, 4°C). A 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 NADPH, 0.1 ml of 0.6 mM DTNB, 0.150 ml of H$_2$O and 50 µl the sample. The cuvette with the mixture was incubated for 5 min at 37°C and then supplemented with 0.6 U GR. The reaction kinetics was followed spectrophotometrically (Pharmacia LKB Ultraspec III, UV/VISIBLE spectrophotometer, Beaconsfield, UK) at 412 nm for 5 min by monitoring the increase in absorbance.

The GSSG concentration was determined in supernatant aliquots by the same method after optimalization of pH to 6–7 with 1 M TEA and derivatization of endogenous GSH with 2-vinylpyridine (v:v). The reduced GSH level in the supernatant 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 for total GSH and 0.975–62 GSSG µM for GSSG).

**Assay of TNF-α concentration**

The pulmonary level of TNF-α was quantified using specific ELISA kits (Quantikine TNF-α, R&D Systems, USA) for rats according to the manufacturer's instructions. The result was read using a TEK Instruments EL340 BIO- spectrophotometer (Winooski, VT, USA) (λ = 450 nm). The TNF-α concentration was read from standard curves and expressed in pg/ml.

**Western blot analysis of p65 subunit of NF-κB**

The expression of p65 was determined in cytosolic fraction and nuclear extracts as previously described (Liu and Lee 2008). To obtain cytosolic fraction 0.1 g tissue was homogenized in 1 ml tissue homogenizing buffer (10 mM TrisHCl (pH 7.5), 1.5 mM MgCl$_2$, 10 mM KCl, 0.3 mM sucrose, 0.1 mM EGTA, 1% Triton-X 100) containing protease and phosphatase inhibitors. After homogenization samples were centrifuged (3,000 rpm, 20 min, 4°C), supernatant was collected and centrifuged (40,000 rpm, 1 h, 4°C). Next, 50 µl was taken for total protein level assay and the remaining supernatant was stored at −80°C until assay. For nuclear extraction pellet from first centrifugation was washed twice in homogenizing buffer and resuspended in nuclear extract buffer (20 mM Tris-HCl (pH 7.6), 40 mM NaCl, 1.5 mM MgCl$_2$, 0.1 mM EGTA, 0.2 mM EDTA, 25% glycerol) with protease and phosphatase inhibitors. The resuspensions were centrifuged (14,000 rpm, 20 min, 4°C), the protein level was assayed and supernatant was stored at −80°C. The total amount of protein in cytosolic and nuclear fraction was determined using the Bradford method. Samples were mixed with Laemmlı’s buffer and boiled for 5 min. Next, equal amounts of protein were loaded on each lane, fractioned by β-tubulin level determination. No β-tubulin was observed in cytoplasmic fraction.

**Statistical analysis**

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

**Results**

**Changes in heart homogenate oxidative injury**

Injection of LPS alone resulted in an elevated amount of the TBARS (p < 0.05) and the H$_2$O$_2$ (p < 0.05) concentration
as compared to the saline group (Table 1). Moreover, it significantly increased protein and TNF-α concentration (p < 0.01 and p < 0.05, respectively) (Figure 1). To determine the role of the ETA-R blockage on LPS-induced oxidative stress we used BQ123 in two doses, 0.5 mg/kg and 1 mg/kg. Our results showed that both doses significantly decreased lipid peroxidation (p < 0.05) but not H2O2 concentration (p > 0.05) in the heart (Table 1). Moreover, BQ123 in a dose of 0.5 mg/kg and 1 mg/kg caused about a 3-fold decrease in the protein level (p < 0.01, p < 0.001, respectively). However, BQ123 only in a dose of 1 mg/kg significantly decreased the TNF-α level endotoxemic hearts (p > 0.05) (Figure 1).

The blockage of ETB-R with BQ788 significantly inhibited an LPS-induced increase in lipid peroxidation (p < 0.05) and H2O2 concentration (p < 0.02). Moreover, it was nearly twice more potent in decreasing protein level after LPS challenge than BQ123 (p < 0.001) (Table 1). However, it had no influence on TNF-α concentration (p > 0.05) (Figure 1).

To measure the participation of ET-1 receptor blockers in modulation of antioxidant properties of the heart tissue under endotoxemia we measured tGSH, GSH and GSSG (Figure 2). LPS administration resulted in a 5-fold decrease in the tissue redox status (p < 0.01) (Table 1), which was connected with a significant (p < 0.01) decrease in tGSH level and nearly a 3-fold depletion of GSH concentration (p < 0.05) (Figure 2). Blockage of ETA-R, but no ETB-R, significantly improved the tissue redox status. BQ123 in a dose of either 0.5 mg/kg or 1 mg/kg caused nearly a 7-fold increase in the GSH/GSSG status (p < 0.01) when compared to the LPS group. Furthermore, it significantly increased the tGSH concentration (p < 0.05) (Figure 2). The GSH level was increased 5.7-fold in both groups when compared to the LPS group.

However, the blockage of ETB-R had little effect on the tissue redox status under endotoxemia (Table 1). BQ788 increased the tGSH concentration (p < 0.05) but it had little influence on the GSH and GSSH concentration (Figure 2).

### p65 subunit concentration

LPS injection caused a 3-fold increase in the p65 subunit level in nuclear fraction when compared with the saline group (44.387 ± 3.347 vs. 13.317 ± 0.792; p < 0.01). Neither blockage of ETA-R and ETB-R before LPS administration influenced significantly the p65 subunit level in the heart homogenates. However, BQ123 at a dose of 1 mg/kg administered before LPS insignificantly decreased p65 subunit level in nuclear fraction (2.524 ± 1.03 vs. 3.333 ± 1.36 in LPS group, p > 0.05).

### Discussion

Our findings suggest that endogenous ET-1 is involved in the production of free radicals during endotoxemia but NF-κB pathway is not involved in this process. Moreover, it highlights the positive, protective role of ETA-R blockage on free radical generation in the heart tissue during LPS-induced endotoxic shock. The blockage of ETA-R decreased free radical generation and improved tissue redox status, while

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline</th>
<th>Saline + LPS</th>
<th>BQ123 (0.5 mg/kg) + LPS</th>
<th>BQ123 (1 mg/kg) + LPS</th>
<th>BQ788 (3 mg/kg) + LPS</th>
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<tbody>
<tr>
<td>TBARS (µM)</td>
<td>33.95 ± 2.37</td>
<td>256.16 ± 11.22*</td>
<td>37.07 ± 3.65*</td>
<td>49.05 ± 13.3*</td>
<td>83.44 ± 16.90*</td>
</tr>
<tr>
<td>H2O2 (µM)</td>
<td>77.96 ± 8.28</td>
<td>225.25 ± 44.76*</td>
<td>118.42 ± 12.13</td>
<td>128.49 ± 4.49</td>
<td>52.72 ± 10.68**</td>
</tr>
<tr>
<td>Protein (µg/ml)</td>
<td>50.3 ± 3.66</td>
<td>89.0 ± 5.84***</td>
<td>33.8 ± 0.84***</td>
<td>28.8 ± 0.8***</td>
<td>19.4 ± 1.5****</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>2.10 ± 0.4</td>
<td>0.33 ± 0.05***</td>
<td>2.19 ± 0.1###</td>
<td>2.32 ± 0.09###</td>
<td>0.57 ± 0.1</td>
</tr>
</tbody>
</table>

The results are mean ± SEM. The data was statistically evaluated by one-way ANOVA. * p < 0.05, *** p < 0.01 vs. saline group; # p < 0.05, ## p < 0.02; ### p < 0.01, #### p < 0.001 vs. saline+LPS (LPS concentration: 15 mg/kg).
the ETB-R blockage decreased free radical generation but had little effect on the heart antioxidant properties. However, neither the ETA-R nor the ETB-R blockage significantly influenced NF-κB p65 subunit in heart homogenates under endotoxemia.

In our study, LPS administration caused an increase in lipid peroxidation products, hydrogen peroxide concentration, as well as a decrease in the GSH/GSSG ratio in the heart homogenates. The increase in ROS production and TNF-α concentration was accompanied by the stimulation of NF-κB pathway.

Oxidative damage is probably one of several factors that lead to cell damage, organ dysfunction, and death. The heart tissue is characterized by high polyunsaturated fatty acids concentration and relatively low amount and activity of antioxidant compounds that make it susceptible to oxidative stress injuries.

Moreover, it has been shown that during endotoxemia a marked increase in superoxide dismutase (SOD) and catalase (CAT) activity lead to overproduction of hydrogen peroxide and superoxide anions resulting in excessive lipid peroxidation in the heart tissue (Ritter et al. 2003). Additionally, LPS was shown to cause a strong inflammatory response that leads to endocardium cells necrosis (de Azevedo et al. 2007).

Previously, Forni et al. (2005) reported that LPS infusion evokes a large increase in plasma concentration of ET-1, and an increase in heart ET-1, ETA-R and ETB-R mRNA level. Therefore, a detrimental effect of endotoxin is partially a result of overproduction of ET-1.

In our study, BQ123 administration prevented LPS-induced increase in lipid peroxidation and a decrease in protein level. It also slightly decreased H2O2 concentration. Wölkart et al. (2006) observed similar effects of BQ123 administration in diabetic animals; in their study ETA-R blockage decreased lipid peroxidation and improved heart antioxidant status. The decrease in hydrogen peroxide concentration and lipid peroxidation after the ETA-R blockage is in part connected with changes in antioxidant enzyme concentration and activity. Ozdemir et al. (2006) showed that BQ123 administration increased SOD, CAT and glutathione reductase (GSHRd) concentration in the ischemic heart. Xu et al. (2003) obtained similar results where the ETA-R blockage but not ETB-R blockage decreased lipid peroxidation and enhanced SOD activity in the ischemic myocardium. It has been also indicated that the ETA-R blockage with BMS 182874 decreased superoxide production in coronary arteries (Elmarakby et al. 2004).

Our results obtained with the ETB-R antagonist, BQ788, suggest that LPS-induced H2O2 generation is mediated by ETB-R rather than ETA-R in heart.

In endothelial cells ETB-R stimulation leads to an increased production of nitric oxide, prostacycline, and endothelial derived hyperpolarizing factor, which cause

Figure 2. Tissue concentration of total (A), oxidized (B) and reduced (C) glutathione concentration in experimental groups: saline, saline + LPS, BQ123 (0.5 mg/kg) + LPS, BQ123 (1 mg/kg) + LPS and BQ788 (3 mg/kg) + LPS (n = 6, per group). Data is shown as mean ± SEM. tGSH, total glutathione; GSH, reduced glutathione; GSSG, oxidized glutathione. * p < 0.05, *** p < 0.01 vs. saline, # p < 0.05, ### p < 0.01, #### p < 0.001 vs. LPS.
vasodilatation. Excessive production of NO during endotoxemia leads to decreased superoxide and therefore, ET-1 production (Callera et al. 2003). Recently, Viola et al. (2007) indicated that \( \text{H}_2\text{O}_2 \) activates calcium channels in ventricular myocytes and increases mitochondrial superoxide production. Hence, in our study a decreased \( \text{H}_2\text{O}_2 \) level after the BQ788 administration is possible to be connected with mitochondrial decrease in calcium uptake.

We further demonstrated that only the ETA-R blockage prevented LPS-induced decrease in tissue redox status. The LPS injection decreased the redox status of the heart tissue and administration of BQ123 but not BQ788 reversed these changes. Therefore, it suggests that ETA-R blockage is more effective in preventing antioxidant properties of the heart tissue under endotoxemia.

Glutathione is a thiol compound and main intracellular antioxidant. Under physiological conditions it scavenges singlet oxygen, superoxide anion or hydroxyl radical and is a source of hydrogen peroxide. LPS-induced cardiomyocyte damage and oxidative stress increase activity of glutathione peroxidase and decrease the concentration of GSH (Motawi et al. 2011). Recently, Goyal et al. (2010) observed that decreased activity of SOD, CAT and GSH level after ET-1 administration was ameliorated by BQ123 treatment.

In our study, neither ETA-R nor ETB-R blockage influenced the LPS-induced increase in NF-κB p65 protein level. However, NF-κB is a crucial integrator of immunological response and it appears to influence the endothelin pathway. NF-κB is known to promote nuclear transcription process, resulting in excessive production of ROS and upregulation of ETA-R. Moreover, our previous study showed that ETA-R blockage significantly decreased NF-κB activation in hearts (Piechota and Goraca 2011). While Xia et al. (2006) indicated that durasentan, an ETA-R inhibitor, downregulates NF-κB expression in heart ventricles. Furthermore, administration of BQ485, ETA-R blocker, reduced ET-1 induced activation of Ras-Raf-MAPK cellular pathway in myocytes inhibiting inflammatory process and cellular damage (Cheng et al. 2005).

**Limitation of the study.** In our study, we measured lipid peroxidation by assaying TBARS level; however, many controversies has appeared in the literature regarding the specificity of TBARS toward compounds other than malondialdehyde. In addition, it was indicated that TBARS

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**Figure 3.** Nuclear assembly of the p65 component in experimental groups: saline, saline + LPS, BQ123 (0.5 mg/kg) + LPS, BQ123 (1 mg/kg) + LPS and BQ788 (3 mg/kg) + LPS \((n = 4, \text{per group})\). A. Representative Western blot analysis. B. Densitometric quantification of p65 subunit of NF-κB in heart tissue. Each column indicates mean ± SEM, OD, optical density. * \( p < 0.05 \), ** \( p < 0.01 \) vs. saline.
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reflects other compounds level such as sialic acid. Sialic acid is a component of glycoproteins and glycolipids which occurs in hormones and enzymes in serum and tissues. Moreover, an increase in sialic acid concentration was found under endotoxia (Gee et al. 2003).

Conclusion

Taking together, our data suggests that under endotoxia ETA-Rblockage is more effective in inhibiting free radical and TNF-α generation and improving heart antioxidant properties than ETB-R blockage. However, neither ETA-R nor ETB-R takes part in LPS-induced activation of NF-κB pathway. These suggest that in the heart tissue ETA-R and ETB-R influence ROS production by some alternative signaling pathways.

Lately, ET-1 receptor blockers have been approved in the treatment of pulmonary hypertension (Saströ 2006), but clinical studies where both selective ETA-R and nonselective ETR antagonists were used in patients with different degrees of heart failure brought mixed results (Abrahams 2001; Kelland and Webb 2006). Therefore, additional knowledge about ET-1 receptor antagonist in regulation of oxidative stress and signaling pathways can provide a survival benefit.

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