INFLUENCE OF NUCLEAR FACTOR-κB INHIBITION ON ENDOTHELIN-1 INDUCED LUNG EDEMA AND OXIDATIVE STRESS IN RATS

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The aim of the present study is to determine the effects of the BAY 11-7082, a nuclear factor-kappaB (NF-κB) inhibitor, on endothelin-1 (ET-1) induced lung edema, the level of reactive oxygen species (ROS) and tumor necrosis factor alpha (TNF-α) in the lungs. Experiments were carried out on adult male Wistar-Kyoto rats. The animals were divided into 4 groups: Group I: saline-treated control; Group II: saline followed by ET-1 (12.5 µg/kg b.w., i.v.); Group III: BAY 11-7082 (10 mg/kg b.w., i.v.) administered 1 hour before saline; Group IV: BAY 11-7082 (10 mg/kg b.w., i.v.) administered 1 hour before ET-1 (12.5 µg/kg b.w., i.v.). Injection of ET-1 alone showed a significant (P<0.001) increase in thiobarbituric acid reactive substances (TBARS) and hydrogen peroxide (H2O2) level as well as a decrease (P<0.01) in GSH level and GSH/GSSG ratio (P<0.02). BAY 11-7082 significantly decreased TBARS (P<0.01) and H2O2 (P<0.05) level as well as improved the redox status (P<0.02) in the lungs. BAY 11-7082 also prevented ET-1 induced lung edema (P<0.05). The concentration of TNF-α (P<0.02) and p65 subunit of NF-κB signaling compound (P<0.001) was increased in the presence of ET-1, while BAY 11-7082 decreased both TNF-α level (P<0.05) and p65 subunit concentration (P<0.01). Our results indicate that BAY 11-7082 plays a protective role in ET-1 induced oxidative lung injury. It successfully prevents lung edema as well as ROS and TNF-α overproduction. Our results also highlight the important role of the NF-κB pathway in ET-1 induced lung injury and ROS overproduction.

Key words: endothelin-1, BAY 11-7082, lung edema, nuclear factor-κB, oxidative stress, tumor necrosis factor alpha, glutathione

INTRODUCTION

Endothelins are a family of 21 amino acid peptides with powerful vasoconstrictive and mitogenic properties (1). The endothelin (ET-1) level in the lung tissue is particularly high compared with other tissues, as their cellular origin in the lungs includes airway epithelial cells and macrophages in addition to vascular endothelial cells (2). The effects of ET-1 are mediated by type A (ETA-R) and type B1 (ETB1-R) receptors, which mediate vasoconstriction, and type B2 (ETB2-R) receptor, which mediates vasodilatation (3). ETα-R and ETβ-R are localized on pulmonary arteries, airway smooth muscles and alveolar wall tissue capillaries, while ETβ1-R is mainly placed on the endothelium (4). An increased blood concentration of ET-1 results in enhanced pulmonary vascular resistance, followed by a decrease in blood flow and eventually development of pulmonary hypertension (5). Fagan et al. reported that ET-1 level in serum and bronchoalveolar lavage (BALF) is significantly higher in patients with acute lung injury, severe asthma, acute respiratory distress syndrome, pulmonary hypertension, and sepsis (6). The latest study performed by Salama et al. indicated that the serum level of ET-1 may be a predictor of bronchiolitis obliterans syndrome (7). Moreover, ET-1 was shown to mediate bronchoconstriction and denudation of the airway epithelium (6). Hence, ET-1 is a significant trigger of inflammatory pathways in lungs (8). The pro-inflammatory action of ET-1 is also associated with the superoxide anion production and TNF-α release. A decrease in the superoxide anion production after ET-R blockade indicates that reactive oxygen species (ROS) are involved in production and action of ET-1 (9).

Improved understanding of biological mechanisms that regulate pro-inflammatory pathways in the lungs could be useful in designing new therapies to limit or prevent lung injury in patients at risk for lung diseases where overproduction of ROS is observed. The molecular signaling pathway by which ET-1 stimulates ROS in the lungs is not thoroughly known. We have chosen to study the NF-κB pathway for a few reasons; firstly, it is one of the major pathways by which ET-1 acts, and the promoter of the ET-1 gene contains a functional NF-κB binding site (10). Additionally, NF-κB is a redox-sensitive transcription factor (11) and generation of ROS seems to lead to IκB degradation and nuclear NF-κB accumulation. Moreover, NF-κB is one of the most important signal transducers of TNF-α (12) and its activation in airway epithelial cells is sufficient for generating acute lung injury (13).

BAY 11-7082, an irreversible inhibitor of IκB phosphorylation and degradation, has been shown to inhibit both the translocation of NF-κB into the nucleus and its subsequent binding to DNA (14). Thus, BAY 11-7082 has been chosen in this study to test whether the ET-1 induced increase in ROS formation and TNF-α release is accompanied by the activation of the NF-κB pathway. Moreover, its inhibitory action on ROS and cytokine release in the lungs remains to be proven.
Chemicals and reagents

Thiobarbituric acid (TBA), butylated hydroxytoluene, 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). All other reagents were obtained from POCH (Gliwice, Poland) and were of analytical grade. Sterile, deionized water was used throughout the study. Endothelin-1 (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 0.9% NaCl. BAY 11-7082 (MERCK, Darmstadt, Germany) was dissolved in 10% DMSO and adjusted to 0.1% solution before intravenous administration.

Animals

The experiments were performed under urethane anesthesia on 32 male Wistar-Kyoto rats weighing 200-230 g, aged 2-3 months. The rats were obtained from the Medical University of Lodz animal quarters and were housed at an ambient temperature of 20±2°C with a 12h light-dark cycle, with free access to food and tap water, until used in the experiments. All animals received a standard laboratory diet and water ad libitum and were maintained for 1 week in the laboratory for adaptation. All experiments were approved by the local Medical University of Lodz Ethics Committee No. 20/L418/2008.

Experimental groups

Animals were assigned randomly to one of the following 4 groups (n=8 per group). In group I (control), rats received 0.2 ml of 0.9% NaCl (i.v.) and 60 min later again 0.2 ml of 0.9% NaCl (i.v.). In group II (ET-1), rats received 0.2 ml of saline (i.v.) and 60 min later 0.2 ml of saline. In group III (BAY-saline), rats received 0.2 ml of BAY 11-7082 (10 mg/kg b.w. i.v.) and 60 min later 0.2 ml of saline. In group IV (BAY-ET-1), rats received 0.2 ml of BAY 11-7082 (10 mg/kg b.w. i.v.) and 60 min later 0.2 ml of ET-1 (12.5 µg/kg b.w. i.v.).

Animal preparations

Animals were anesthetized by an intraperitoneal injection of 10% urethane (2 ml/100 g b.w.). When a sufficient level of anesthesia was achieved, the skin and subcutaneous tissues on the neck were infiltrated with 2% polocaine hydrochloric solution (Polfa, Poland) and a 2-cm-long polyethylene tube (2.00 mm O.D.) was inserted into the trachea. The right femoral artery was catheterized for drug infusion. All drugs were administered directly into the femoral vein. Animals were euthanized under anesthesia 5 hours after saline or ET-1 administration. The thoracic cavity was opened to remove lungs. The left lung (about 500 mg) was cut off, weighed and frozen in -80°C until used for measurements of the oxidative stress parameters, TNF-α concentration and NF-kB p65 subunit concentration. The right lung (about 500 mg) was cut off and used immediately for the lung edema assay.

Lung edema assay

The lungs were rinsed with ice-cold saline, weighed (wet weight) and placed in a drying oven at 80°C for 8 h, before being weighed again (dry weight). Finally, the lung wet-to-dry weight ratios were determined as an indicator of lung edema (15).

Measurement of thiobarbituric acid reactive substance (TBARS) level

The lipid peroxidation product content in the lung homogenates was assayed for TBARS, previously described by Yagi et al. (16) with some modifications. Briefly, homogenate samples were incubated for 15 min at 95°C in a mixture (0.015% butylated hydroxytoluene, 0.375% thiobarbituric acid, and 15% trichloroacetic acid) and then centrifuged with 2.5 ml of butanol at 3,000 r.p.m. for 10 min. Thiobarbituric acid reactive substances were measured spectrophotometrically using a Perkin Elmer Luminescence Spectrometer LS-50 (Norwalk, CT, U.S.A.). Excitation was set at 515 nm and emission was measured at 546 nm. Readings were converted into μM using the calibration curve obtained for tetramethoxypropane (0.01-50 μM). Finally, the results were calculated for 50 mg of lung tissue.

Determination of H2O2

Generation of H2O2 in lung homogenates was determined according to the Ruhs et al. method (17). H2O2 concentration was measured in homogenate samples using HRP/HVA systems. Samples were incubated for 60 min at 37°C and the enzymatic reaction was stopped by adding 0.1 M glycine-NaOH buffer (pH 12.0) with 25 mM EDTA. Excitation was set at 312 nm and emission was measured at 420 nm (Perkin Elmer Luminescence Spectrometer, Beaconsfield UK). Readings were converted into H2O2 concentration using a standard curve for 10 increasing H2O2 concentrations (range 10-1000 μM).

Determination of protein concentration

Protein content in the lung homogenates was measured with the spectrophotometric method described by Lowry et al. (18). The values for absorbance at 750 nm were converted to protein concentration using a standard curve for 10 increasing bovine serum albumin concentrations (10-37.5 μg/ml).

Determination of glutathione levels

Total glutathione (tGSH), reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in lung homogenates. Briefly, lungs were homogenized in cold 5% 5-SSA and centrifuged (10,000 x g, 10 min, 4°C). The resulting supernatant was divided between two Eppendorf tubes. In the first tube, a mixture of 0.2 mM NADPH and 0.1 ml of 0.6 mM DTNB was added to assay total glutathione, while the same mixture supplemented with 1 M TEA and 2-vinylpyridine was added to the second tube to assess the level of GSSG. Next, 0.6 U of GR was added to trigger a reaction. The reaction kinetics was followed spectrophotometrically by monitoring the increase in absorbance at 412 nm for 5 min. The reduced supernatant GSH level was calculated as the difference between tGSH and GSSG. The increments in absorbance at 412 nm were converted to GSH and GSSG concentrations using a standard curve (3.2-500 μM of GSH for total GSH and 0.975-62 μM of GSSG for GSSG). The results were expressed in μM.

Tumor necrosis factor-α assay

TNF-α in the lung tissue was assayed by specific enzyme-linked immunosorbent assay using a commercially-available ELISA test kit (R&D Systems) containing a monoclonal antibody specific for rat TNF-α. The result was read using a TEK Instruments EL340 BIO- spectrophotometer (Winooksi, VT, USA) (λ=450 nm). The TNF-α concentration was read from standard curves and expressed in pg/ml.
Western blot determination of p65 of nuclear factor-κB in lung tissue

Nuclear extracts were prepared from the lungs using the RIPA buffer as previously described (19). The total amount of protein was determined using the Lowry’s method. The immunoblot analysis was performed according to Laemmli (20). The detection of p65 activation was performed using primary antibodies for p65 (Santa Cruz Biotechnology 1:600 dilution) and goat anti-rabbit IgG-horseradish peroxidase antibodies (Santa Cruz Biotechnology 1:1000 dilution). The protein bands were revealed using chromometry, and their abundance was analyzed by scanning the exposed films densitometrically using an Image Master VDS (Pharmacia Biotech) with appropriate software.

Statistical analysis

The results were analyzed with Statistica 8.0 software. ANOVA followed by Tukey’s tests as post-hoc was applied. Data are presented as mean±S.E.M. P≤0.05 is regarded as being statistically significant.

RESULTS

Effects of endothelin-1 and BAY 11-7082 on protein level and lung edema

The development of lung edema was measured as the wet-to-dry ratio (W/D ratio). The lung tissue W/D ratio analysis demonstrated 3.9-fold higher (P<0.001) water content in the ET-1 group. BAY administration markedly (P<0.05) decreased the ET-1 induced increase in the W/D ratio compared with the ET-1 group (Table 1). Despite a significant increase in the W/D ratio after ET-1 infusion there was an insignificant decrease in the protein level after ET-1 administration compared with the control (P>0.05). However, BAY decreased a 3-fold protein level when administered before ET-1 (P<0.001) (Table 1).

endothelin-1 and BAY 11-7082 effects on lipid peroxidation and $H_2O_2$ level

TBARS content in the lungs increased almost 3-fold (P<0.001) after ET-1. BAY alone did not markedly influence (P>0.05) lipid peroxidation, while it caused a significant (P<0.01) reduction in the TBARS content when administered before ET-1 (Table 1). Furthermore, ET-1 intravenous administration led to a significant (P<0.001) 4.3-fold increase in $H_2O_2$ level compared with the control. ET-1 induced increase in $H_2O_2$ level was also significantly (P<0.05) prevented by BAY (Table 1).

Table 1. The influence of endothelin-1 on thiobarbituric acid reactive substance (TBARS) level, hydrogen peroxide ($H_2O_2$) concentration, protein level and wet-to-dry (W/D) ratio in lungs. The results are mean ±S.E.M. The data was statistically evaluated by one-way ANOVA.

<table>
<thead>
<tr>
<th>Condition</th>
<th>TBARS (μM)</th>
<th>$H_2O_2$ (μM)</th>
<th>Protein (μg/ml)</th>
<th>W/D ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl + 0.9% NaCl + ET-1 (n=8)</td>
<td>48.4±2.39</td>
<td>31.99±3.54</td>
<td>30.6±3.17</td>
<td>4.8±1.46</td>
</tr>
<tr>
<td>0.9% NaCl + ET-1 (12.5 μg/kg) (n=8)</td>
<td>144.25±10.55***</td>
<td>140.19±17.82***</td>
<td>24.25±4.95</td>
<td>8.7±1.77</td>
</tr>
<tr>
<td>BAY 11-7082 (10 mg/kg) + 0.9% NaCl (n=8)</td>
<td>58.31±3.91</td>
<td>27.31±9.68</td>
<td>18.42±1.89***</td>
<td>9.20±0.8</td>
</tr>
<tr>
<td>BAY 11-7082 (10 mg/kg) + ET-1 (12.5 μg/kg) (n=8)</td>
<td>75.47±8.64*** ***</td>
<td>64.25±18.45*</td>
<td>10.35±2.53****</td>
<td>12.40±1.36*</td>
</tr>
</tbody>
</table>

* p<0.05; ** p<0.02; *** p<0.01; **** p<0.001 vs. control; # p<0.05; ## p<0.02; ### p<0.01; #### p<0.001 vs. ET-1

Assay of redox status in the lungs

A significant 2.8-fold (P<0.001) reduction in tGSH concentration and 4.3-fold decrease in the GSH/GSSG ratio (P<0.02) was observed after ET-1 infusion. The decreased GSH/GSSG ratio induced by ET-1 suggested that the balance of the GSH system changed in favor of oxidation. The IκBκ inhibitor significantly augmented intracellular GSH (P<0.001) and GSH (P<0.01) levels, as well as decreased GSSG concentration (P<0.02) when compared to ET-1 group. Furthermore, in the BAY+ET-1 group, the GSH/GSSG ratio increased 2.8-fold (P<0.02) (Fig. 1A and B).

Determination of tumor necrosis factor-α

Fig. 2 shows that 5 hours after ET-1 administration, the lung concentration of TNF-α increased by 33% (P<0.02). Intravenous BAY administration significantly prevented ET-1 induced TNF-α augmentation (P<0.05).

Effects of endothelin-1 and BAY 11-7082 on p65 subunit concentration

Since NF-κB regulates the expression of pro-inflammatory genes, the content of the p65 subunit of NF-κB in the nuclear fraction was chosen as an indicator of its activation. ET-1 caused a 7-fold increase in p65 concentration compared with the control (P<0.001). Moreover, BAY significantly (P<0.01) decreased the ET-1 induced increase in the p65 subunit level (Fig. 3A and B) compared with the ET-1 group. These results may suggest that ET-1-induced ROS generation may depend on activation of NF-κB in the lung tissue.

DISCUSSION

The major findings of the present study are that: (i) endothelin-1 leads to lung edema development, which is significantly suppressed by BAY 11-7082, an irreversible inhibitor of IκBκ phosphorylation and degradation; (ii) BAY 11-7082 significantly prevents ET-1-induced decrease in lung antioxidant ability and it decreases free radicals and TNF-α level; (iii) endothelin-1 activates NF-κB pathway in rat lungs.

The present study showed a significant increase in the TBARS and $H_2O_2$ concentrations as well as a decrease in GSH and GSH/GSSG ratio in lung homogenates following the administration of exogenous ET-1. This is consistent with previous reports, which demonstrated that ET-1 may lead to oxidative stress by reducing the antioxidant GSH/GSSG ratio and stimulating lipid peroxidation in a time-dependent manner (21). In addition, Wadgewood et al. reported that ET-1 increased
superoxide production via ETA-R in pulmonary artery smooth muscle cells (22).

Recently, Di Giulio et al. (23) indicated that ET-1 is involved in the carotid body chemosensory responses to hypoxia in rats. Hypoxia may contribute to pulmonary vessels constriction and the development of pulmonary hypertension which is accompanied by higher ET-1 level (6, 24).

To test whether ET-1 contributes to lung edema formation, we have measured the wet-to-dry ratio and protein concentration in lungs. ET-1 was successful in lung edema development that was not followed by an increase in protein concentration in lung tissue. However, it has been demonstrated by Berger et al. (25) that ET-1 contributes to the lung edema formation connected with enhanced permeability for water and small molecules but not for albumin. Moreover, when ET-1 combines to ET A-R it increases vascular endothelial growth factor (VEGF) content. VEGF promotes vascular angiogenesis and increases vascular permeability. It has been shown that VEGF markedly reduces pulmonary vascular protein extravasation in the hypoxic ETB-R–deficient rats (26).

Fig. 1. Total oxidized and reduced glutathione concentration (A) and glutathione redox ratio (B) in lung homogenates in control, after endothelin-1 (12.5 µg/kg b.w.), BAY 11-7082 (10 mg/kg b.w.) and BAY 11-7082 + endothelin-1 (10 mg/kg b.w. and 12.5 µg/kg b.w., respectively) (n=8, per group). Data is shown as mean ±S.E.M. * p<0.05; ** p<0.02; *** p<0.001 vs. control; # p<0.01 vs. ET-1.

Fig. 2. TNF-α concentration in lung homogenates in control, after endothelin-1 (12.5 µg/kg b.w.), BAY 11-7082 (10 mg/kg b.w.) and BAY 11-7082+endothelin-1 (10 mg/kg b.w. and 12.5 µg/kg b.w., respectively) (n=8, per group). Data is shown as mean ±S.E.M. ** p<0.02 vs. control; # p<0.05 vs. ET-1.
In our studies, ET-1 intensified TNF-α level in lung homogenates. This finding is consistent with other authors who have shown that overexpression of ET-1 took part in TNF-α, IL-1 and IL-6 upregulation as well as intravascular macrophage accumulation and monocyte adherence in the lung (25). Furthermore, TNF-α can influence monocyte adhesion directly and can influence the ETαR expression and NO production in the lung endothelial cells (27).

Our study has also shown that BAY 11-7082 effectively decreased the lung edema formation and protein concentration in the lung tissue. Recently, Sarada et al. (28) reported that curcumin (a yellow pigment which has the ability to downregulate the NF-κB activation) reduced the transvascular leakage in the lungs of hypoxic exposure rats. This blocker also down-regulated the activation of NF-κB. Moreover, Cheng et al. demonstrated that NF-κB signaling in airway epithelial cells led to increased vascular permeability in the lungs, and the inhibition of NF-κB activation reduced lung inflammation and edema (15).

Our results also demonstrated that BAY 11-7082 significantly decreased TBARS and H2O2 and increased the GSH/GSSG ratio, thus indicating a reduction in ROS creation. Moreover, BAY 11-7082 efficiently suppressed ET-1 induced TNF-α increase and NF-κB activation, reflected by decreased NF-κB p65 subunit concentration in the nuclear fraction. Overproduction of TNF-α under oxidative stress conditions is thought to be connected with the activation of the NF-κB pathway and TNF-α. Therefore, it seems likely that ET-1, which evokes ROS generation, also leads to TNF-α secretion by the NF-κB pathway. Hence, the beneficial effects of BAY 11-7082 in the lung tissue might be associated with a reduced NF-κB activation.

However, the nature of the mechanism inhibiting NF-κB activation is unclear. It has been previously indicated that the effect of NF-κB inhibition on ROS and cytokine production is likely to differ depending upon the incubation time, concentration of NF-κB inhibitors, and cell type (27, 29). Postea et al. (30) showed that inhibition of NF-κB activation by BAY 11-7082 abolished homocysteine-induced generation of ROS and NF-κB translocation to the nucleus. Rahman et al. (31) indicated that direct and indirect oxidative stress in airway epithelium and alveolar macrophages stimulate cytokines such as TNF-α, which in turn can activate the NF-κB pathway in airway epithelial cells and stimulate pro-inflammatory genes for IL-1, IL-6, IL-8, TNF-α and genes for antioxidant enzymes such as SOD. This may lead to stimulation of ROS and RNS production and eventually to a decrease in the antioxidant enzyme concentration. Moreover, BAY 11-7082 was shown to actively decrease the formation of pro-inflammatory cytokines such as TNF-α (14). In in vitro study, it was indicated that NF-κB mediates cell survival through suppression of the accumulation of ROS (32).

In summary, the present study has demonstrated that BAY 11-7082, due to its antioxidant activities and NF-κB inhibition, can significantly prevent ET-1 induced lung edema as well as ROS and TNF-α generation in lungs. Moreover, our studies highlight ET-1 role in the activation of NF-κB and stimulation of oxidative stress in the lungs of rats.

Our findings have potential clinical implications, as patients with pulmonary disorders like acute respiratory diseases have higher levels of ET-1, which could be pharmacologically modulated with NF-κB pathway inhibitors.

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REFERENCES


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