Nuclear factor κB inhibitor BAY 11-7082 suppresses oxidative stress induced by endothelin-1 (ET-1) in rat kidney*

Summary

The aim of the study was to evaluate the effect of BAY 11-7082, an NF-κB inhibitor, on basal and ET-1-induced production of reactive oxygen species (ROS), TNF-α and p65 protein in rat kidney.

Material/Methods:

The experimental animals were divided into five groups (n=7) receiving: 1) saline (control); 2 and 3) ET-1 in a dose of 3 µg/kg body weight (b.w.) or 12.5 µg/kg b.w.; 4) BAY 11-7082 (10 mg/kg b.w.) and ET-1 (12.5 µg/kg b.w.), respectively. In kidney homogenates the concentration of thiobarbituric acid reactive substances (TBARS), H₂O₂, TNF-α, p65 protein and GSH/GSSG ratio were determined.

Results:

ET-1 resulted in a dose-dependent increase in TBARS and hydrogen peroxide (H₂O₂) levels, and a decrease in GSH/GSSG ratio when compared to the controls. BAY 11-7082 administered 1 h before ET-1 administration at a dose of 12.5 µg/kg resulted in a decrease (P<0.001) in TBARS and H₂O₂ levels and an increase (P<0.001) in GSH/GSSG ratio compared to the ET-1 groups. The level of TNF-α was increased (P<0.001) in the presence of ET-1, while BAY 11-7082 reduced the TNF-α level (P<0.001). The rats receiving BAY 11-7082 showed a decrease in NF-κB p65 protein level in the nuclear fraction and an increase in the cytoplasmic fraction.

Conclusions:

The results suggest that BAY 11-7082 plays a protective role against ET-1 induced oxidative stress in kidney tissue. These actions of BAY 11-7082 may result from reduced activity of NF-κB signaling pathways. Inhibition of the NF-κB pathway may be a promising strategy for preventing the progression of kidney damage.

Keywords: oxidative stress • endothelin-1 • NF-κB • p65 protein • BAY 11-7082.

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Introduction

The endothelin (ET) family comprises ET-1, ET-2, and ET-3, which are 21-amino acid peptides derived from distinct genes [32]. The ET isoforms mediate their physiological effects through two endothelin receptors, the endothelin A (ET<sub>A</sub>) receptor and endothelin B (ET<sub>B</sub>) receptor [24]. One of the three peptides, endothelin-1 (ET-1), is a multifunctional peptide that primarily acts as a potent vasoconstrictor with direct effects on the systemic vasculature. ET-1 is the major endothelial isoform in the human kidney and the only one so far shown to be expressed at the protein level. Although most vascular production occurs within endothelial cells, it is also produced by other cell types including vascular smooth muscle cells, epithelial and epicardial cells, fibroblasts, macrophages in the systemic vasculature and arterioles of the kidney [29,30]. Within the renal system, it is produced by glomerular epithelial and mesangial cells, renal tubular and medullary collecting duct cells [17], and resident and infiltrating macrophages. ET-1 plays an important role in cell proliferation, podocyte dysfunction, extracellular matrix remodeling, inflammation and renal fibrosis, and, arguably, these effects may be more significant in the progression of chronic kidney diseases than its prohypertensive activities [8]. Chronic infusion of exogenous ET-1 was found to increase glomerular permeability to albumin, but has no action on proteinuria [1]. Schildroth et al. [25] note that ET-1 increases vascular resistance and the decrease in blood flow contributes to renal tissue dysfunction.

Some authors have indicated that ET-1 plays a role in reactive oxygen species (ROS) formation [5,26]. In vitro studies have shown that ET-1 increases ROS formation in endothelial cells [9], vascular smooth muscle cells [26], rat aortic rings [33], and normal and diabetic glomeruli [21]. ROS, particularly superoxide anion, contribute to acute renal vasoconstriction induced by the ET-1 elicited by both ET<sub>A</sub> and ET<sub>B</sub> receptors. The effects of ROS may be mediated in part by such redox-sensitive proteins as the transcription nuclear factor (NF-κB).

Transcription nuclear factor-kappa B (NF-κB) is primarily involved in immune, inflammatory and stress responses. This factor is found in the cytoplasm of most cells as a dimer bound to an inhibitory protein IκB to form an inactive protein complex that prevents nuclear translocation [13]. Various stimuli, including pro-inflammatory cytokines, activate kinases referred to as IκB kinases (IKKs), which phosphorylate IκBα [18]. Phosphorylation of IκB followed by dissociation and subsequent proteolytic degradation results in the release of NF-κB protein, which translocates to the nucleus and enhances the transcription of many inflammatory genes such as cytokines, chemokines and adhesion molecules [4].

The family of NF-κB transcription factors consists of five members found in mammalian cells: RelA (also referred to as p65), RelB, c-Rel, p50/p105, and p52/p100. These factors are frequently found in heterodimeric or homodimeric complexes. NF-κB is a heterodimer consisting of two subunits, RelA/p65 and p50, and is involved in the regulation of a variety of physiological processes including differentiation, proliferation, inflammation and survival [3,6]. The role of the NF-κB pathway in the mechanism of ROS generation induced by ET-1 administration has not been well characterized. BAY 11-7082 ((E)-3-(4-methylphenylsulfonyl)-2-propenenitrile) is an IκB-α phosphorylation inhibitor, which selectively stops NF-κB activation. So far the effects of BAY 11-7082 on oxidative stress parameters induced by ET-1 administration in the kidney are little known. Therefore the aim of the study was to evaluate the influence of BAY 11-7082 on basal and ET-1-induced production of ROS, TNF-α and p65 protein in kidney homogenates.

Materials and methods

Chemicals

Endothelin-1 (powder), thiobarbituric acid (TBA), sodium acetate trihydrate, butylated hydroxytoluene (BHT), triethanolamine hydrochloride (TEA), 5-sulfosalicylic acid hydrate (5-SSA), 5,5’-dithio-bis (2-nitrobenzoic acid) (DTNB), glutathione reductase (GR), β-NADPH (β-nicotinamide adenine dinucleotide phosphate), 2-vinylpyridine, and 1,1,3,3-tetramethoxypropane (dimethyl acetal) were purchased from SIGMA Chemical Co. (St. Louis, MO, USA). BAY 11-7082 was purchased from MERCK, Darmstadt (Germany). All other reagents were obtained from POCH (Gliwice, Poland).
was dissolved in 10% DMSO and adjusted to 0.1% solution before intravenous administration. Endothelin-1 was dissolved in 0.9% NaCl.

Animals

The experiments were performed on male Wistar rats. Animals were 8–12 weeks old at the time of testing and were acquired from the Medical University of Lodz animal quarters. Laboratory rats were housed in individual cages under standard laboratory conditions: 12/12 h light-dark cycle (light on at 7.00 a.m.) at 20 ± 2°C ambient temperature and 55 ± 5% air humidity. All rats received a standard laboratory diet and water ad libitum and they were maintained for 1 week in the laboratory for adaptation. 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. 20/418/2008.

Experimental design

The experimental animals were divided into five groups, each group comprising seven rats: Group 1 (control rats) received two doses of 0.2 ml saline (1 h apart). Group 2 (ET-1, 3 µg/kg) received one dose of 0.2 ml saline and ET-1 at a dose of 3 µg/kg b.w. 1 h later. Group 3 (ET-1, 12.5 µg/kg) received one dose of 0.2 ml saline followed by ET-1 at a dose of 12.5 µg/kg b.w. 1 h later. Group 4 (BAY 11-7082) received one dose of 0.2 ml saline and the inhibitor of IκBα phosphorylation BAY 11-7082 (10 mg/kg b.w.) 1 h later. Group 5 (BAY 11-7082 + ET-1) received BAY 11-7082 (10 mg/kg b.w.) and ET-1 at a dose of 12.5 µg/kg b.w. 1 h later [23]. All compounds were administered into the femoral vein.

Preparation of animals

The animals were anaesthetized by an intraperitoneal injection (i.p.) of 10% urethane (2 ml/100 g b.w.). Then, the skin and subcutaneous tissues on the neck were cut and a 2 cm-long polyethylene tube (2.00 mm O.D.) was inserted into the trachea. The right femoral vein was catheterized for drug infusion. Rats were euthanized by cervical decapitation 5 h after the administration of the last compound. Kidney tissues were excised, rinsed with ice-cold saline, dried by blotting between two pieces of filter paper, weighed on an electronic scale and then stored at -76°C for measurement of oxidative stress parameters (concentrations of glutathione, H₂O₂ and lipid peroxidation products), levels of TNF-α and the p65 subunit.

Preparation of kidney tissue homogenates

50 mg of tissue was homogenized in either 0.15 M KCl for the estimation of lipid peroxidation products content and H₂O₂ level, or in 5% SSA for the estimation of total, reduced and oxidized glutathione (tGSH, GSH, GSSG). Homogenates were centrifuged at 1 500 x g for 15 min at 4°C, for lipid peroxidation assay and at 6 700 x g for 10 min at 4°C for glutathione measurement. The resulting supernatant was used for biochemical analysis immediately.

Determination of lipid peroxidation products content (TBARS)

The formation of TBARS was used to quantify the degree of lipid peroxidation in kidney tissues. The peroxidation product content was assayed as the concentration of TBARS in the butanol layer, measured spectrophotometrically (excitation 515 nm; emission 546 nm) using an LS-50 Perkin Elmer Luminescence Spectrometer (Norwalk, CT, U.S.A.). The TBARS concentration in the sample was calculated using a regression equation prepared from triplicate assays of six increasing concentrations of tetramethoxypropane (dimethyl acetal; range 0.01–50 µM) as a standard for TBARS. Finally, the results were calculated for 50 mg of the kidney tissue. The results are expressed in µM.

Determination of H₂O₂ concentration

The H₂O₂ concentration in homogenates was measured using horseradish peroxidase/homovanillic acid (HRP/HVA) system. Samples were incubated for 60 min at 37°C, after which the enzymatic reaction was stopped by adding 0.1 M glycine-NaOH buffer (pH 12.0) with 25 mM EDTA. The H₂O₂ concentration was measured spectrophotometrically (excitation 312 nm; emission 420 nm; Perkin Elmer Luminescence Spectrometer, Beaconsfield UK). The readings were converted into a value for the H₂O₂ level using a regression equation prepared from three series of calibration experiments with 10 increasing H₂O₂ concentrations (range 10-1000 µM). The results are expressed in µM.

Determination of glutathione levels

Total (tGSH), reduced (GSH) and oxidized (GSSG) glutathione concentrations were measured in the kidney tissue homogenates. The GSH content of the supernatant was measured in a 1 ml cuvette containing 0.1 ml of 0.6 mM DTNB, 0.7 ml of 0.2 mM NADPH, 0.150 ml of H₂O₂ and 50 µl of the sample. The cuvette with the mixture was incubated for 5 min at 37°C and then supplemented with 0.7 U of glutathione reductase (GR). The reaction kinetics was traced spectrophotometrically at 412 nm for 5 min by monitoring the increase in absorbance. GSSG concentration was determined in supernatant aliquots using the same protocol after optimization of pH to 6-7 with 1 M TEA, and endogenous GSH was determined with 2-vinylpyridine (v,v). The reduced GSH level in the supernatant 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 tGSH and 0.975–60 µM of GSSG for GSSG). The results are expressed in µM.
GSH/GSSG ratio

The redox ratio of each sample was calculated by dividing its reduced glutathione content by the oxidized glutathione content.

Tumor necrosis factor-α (TNF-α) assay

The concentration of TNF-α in the kidney homogenates was measured using an enzyme-linked immunosorbent assay (ELISA) commercial kit (Quantikine, R&D Systems, USA) according to the manufacturer’s instructions. The results were read using a TEK Instruments EL340 Bio-spectrophotometer (Winooski VT, USA) (λ = 450 nm). The results are expressed in pg/ml.

Nuclear factor-κB (NF-κB) p65 subunit assay

The NF-κB p65 subunit concentrations were determined using a specific RELA ELISA kit (ABNOVA, Cat # KA1394 V.01, USA) which measures free p65 protein in the cytoplasmic and nuclear fractions. It was assumed that the nuclear level of p65 correlates positively with the activation of the NF-κB pathway. A plate coated with the anti-p65 antibody was used to capture free p65. The analysis was performed according to the manufacturer’s instructions. The results are expressed in ng/ml.

Results

Effect of ET-1 and BAY 11-7082 on oxidative stress parameters

The changes in oxidative damage parameters are presented in Table 1. The administration of ET-1 at doses of 3 and 12.5 µg/kg resulted in significantly higher TBARS (P < 0.001) levels than those of the control group. Intravenous administration of ET-1 at both doses (3 or 12.5 µg/kg) resulted in significant decreases in tGSH (P < 0.01; P < 0.001, respectively) and GSH levels (P < 0.001) as compared to saline-treated rats (Table 2). BAY 11-7082 (10 mg/kg) administered 1 hour before ET-1 at a dose of 12.5 µg/kg, significantly reduced TBARS (P < 0.001) and \( \text{H}_2\text{O}_2 \) (P < 0.001) levels in the kidney tissue, compared to ET-1 in a dose of 12.5 µg/kg (Table 1). Treatment of rats with BAY 11-7082 (10 mg/kg) and endothelin-1 (12.5 µg/kg) significantly enhanced the ET-1-induced decrease in tGSH and GSH levels (P < 0.001) (Table 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline</th>
<th>ET-1 (3 µg/kg)</th>
<th>ET-1 (12.5 µg/kg)</th>
<th>BAY-11-7082</th>
<th>BAY + ET-1 (12.5 µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS (µM)</td>
<td>11.84 ± 0.58</td>
<td>16.93 ± 0.58 *</td>
<td>22.49 ± 0.53 *</td>
<td>9.77 ± 0.87</td>
<td>11.84 ± 1.05 ✡ ✡</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 ) (µM)</td>
<td>0.19 ± 0.04</td>
<td>0.34 ± 0.04 ***</td>
<td>0.37 ± 0.03 **</td>
<td>0.06 ± 0.02</td>
<td>0.07 ± 0.03 ✡ ✡</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>17.43 ± 1.83</td>
<td>29.84 ± 1.72 ***</td>
<td>52.66 ± 1.25 *</td>
<td>19.18 ± 1.55</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM for groups of seven rats in each. Statistical significance was determined by one-way ANOVA followed by post hoc LSD test. Values are statistically significant at P < 0.05. TBARS - thiobarbituric acid-reactive substances; \( \text{H}_2\text{O}_2 \) - hydrogen peroxide; TNF-α - tumor necrosis factor alpha. *P < 0.001, **P < 0.01, ***P < 0.05 versus saline; ^P < 0.001, ^^P < 0.01, ^^^P < 0.05 versus ET-1 (3 µg/kg); #P < 0.001 versus ET-1 (12.5 µg/kg).

Table 2. Renal state of glutathione metabolism in the control (saline) and after administration of endothelin-1, BAY 11-7082 and BAY + ET-1 (12.5 µg/kg)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline</th>
<th>ET-1 (3 µg/kg)</th>
<th>ET-1 (12.5 µg/kg)</th>
<th>BAY-11-7082</th>
<th>BAY + ET-1 (12.5 µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tGSH (µM)</td>
<td>5.79 ± 0.29</td>
<td>4.42 ± 0.19 **</td>
<td>3.88 ± 0.12 *</td>
<td>7.1 ± 0.16</td>
<td>6.82 ± 0.2 ✡</td>
</tr>
<tr>
<td>GSSG (µM)</td>
<td>1.89 ± 0.1</td>
<td>2.45 ± 0.27</td>
<td>3.37 ± 0.21 *</td>
<td>1.75 ± 0.15</td>
<td>1.37 ± 0.11 ^ ^</td>
</tr>
<tr>
<td>GSH (µM)</td>
<td>3.41 ± 0.39</td>
<td>0.77 ± 0.18 *</td>
<td>0.69 ± 0.08 *</td>
<td>5.32 ± 0.21</td>
<td>5.29 ± 0.23 ^</td>
</tr>
<tr>
<td>GSH/GSSG ratio</td>
<td>1.97 ± 0.3</td>
<td>0.82 ± 0.19 ***</td>
<td>0.75 ± 0.11 **</td>
<td>3.05 ± 0.21</td>
<td>3.45 ± 0.34 ^ ^</td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM for groups of seven rats in each. One-way ANOVA followed by post hoc test LSD. Values are statistically significant at P < 0.05. tGSH - total glutathione (GSSG + GSH); GSH - reduced glutathione; GSSG - oxidized glutathione; GSH/GSSG ratio reduced glutathione/oxidized glutathione. *P < 0.001, **P < 0.01, ***P < 0.05 versus saline; ^P < 0.001, ^^^P < 0.05 versus ET-1 (3 µg/kg); #P < 0.001, #^P < 0.01, ###P < 0.05 versus ET-1 (12.5 µg/kg).
Effect of ET-1 and BAY 11-7082 on redox state

The redox state (GSH/GSSG ratio), an oxidative stress indicator, was found to be significantly lower in the ET-1 (12.5 µg/kg) group than the controls (P < 0.01). However, it was significantly higher in the BAY 11-7082 + ET-1 group compared to the ET-1 (12.5 µg/kg) group (P < 0.001) (Table 2).

Effect of ET-1 and BAY 11-7082 on TNF-α level

Table 1 shows that five hours after ET-1 administration at doses of 3 and 12.5 µg/kg, the renal concentration of TNF-α was significantly higher than that of the control group (P < 0.05; P < 0.001, respectively). By contrast, the level of renal TNF-α was significantly lower (P < 0.001) after administration of BAY 11-7082 alone or combined as BAY 11-7082 + ET-1 compared to ET-1 (12.5 µg/kg) (Table 1).

Effect of ET-1 and BAY 11-7082 on p65 subunit level

Figures 1 and 2 show the changes of NF-κB p65 subunit levels in cytoplasmic and nuclear fractions isolated from kidney tissue. A significantly higher level of p65 protein content was noted in the cytoplasmic and nuclear fractions (P < 0.01) after administration of ET-1 (12.5 µg/kg) alone compared to the control. BAY 11-7082 infusion alone caused a decrease in p65 protein levels in the nuclear fraction (P < 0.05) compared to ET-1 (12.5 µg/kg) administration. Inhibition of IkBα degradation with BAY 11-7082 before ET-1 (12.5 µg/kg) administration caused a decrease in p65 protein level in the nuclear fraction (P < 0.01), compared to ET-1 (12.5 µg/kg). The decreased p65 accumulation in the nucleus was associated with its increased level in the cytoplasm (P < 0.01), indicating inhibition of the NF-κB activation signaling pathway.

Discussion

The results of this study demonstrate that ET-1 administration results in increased production of ROS and the development of oxidative stress in kidney tissue. The oxidative damage of kidney tissue was indicated by increased TBARS, H$_2$O$_2$, and TNF-α levels and a reduction in the GSH/GSSG ratio. Another effect of ET-1 administration was the production of enhanced NF-κB p65 protein levels in both fractions, but significantly higher in the nuclear fraction. It has been reported that blocking the endothelin system using selective and non-selective receptor blockers, as well as drugs reducing ET-1 production, is efficient in reducing the damage to the kidney caused by excessive amounts of ROS [2,15,22]. ROS can directly cause renal parenchymal and microcirculatory damage and result in functional dysregulation with a feedforward loop of hypoxia and ROS generation [12]. Increased ROS generation in our study is supported by the increased lipid peroxidation and level of H$_2$O$_2$. Lipid peroxidation of unsaturated fatty acids impairs cell membrane fluidity and alters the activity of membrane-bound enzymes and receptors [7,27]. H$_2$O$_2$ is an important component in the cascade of events during which ROS are produced, and lipids are oxidized to their hydroperoxy products in the presence of iron, leading to kidney damage.

Glutathione is a primary non-enzymatic intracellular antioxidant. It mainly occurs in a reduced state (GSH) but is oxidized to disulfide glutathione (GSSG) to inactivate free radicals. In this study, the decreased GSH level and GSH/GSSG ratio seen after ET-1 administration reflected the diminished antioxidant status of the tissue and were associated with the increased TBARS level. These results are in line with our previous reports, which demonstrated that ET-1 may lead to oxidative
stress by reducing glutathione, diminishing the antioxidant GSH/GSSG ratio and stimulating lipid peroxidation [16,23].

TNF-α is an inflammatory cytokine that acts mainly through the activation of nuclear factor-κB, contributing to greater renal damage [10]. In experimental models of inflammatory diseases, NF-κB is activated and regulates the expression of genes involved in tissue inflammation such as TNF-α [28]. In the present study, ET-1 administration resulted in an increase in TNF-α level in the kidney tissue, which can further exacerbate the inflammatory damage. Moreover, TNF-α can additionally trigger NF-κB activation. These findings are in agreement with those showing that ET-1 administration stimulates the release of TNF-α and increases the plasma or tissue concentration of TNF-α [14,28].

The NF-κB pathway was chosen as the subject of this study as it is one of the major pathways by which ROS acts, and the promoter of the endothelin gene contains a functional NF-κB-binding site. ET-1 was found to cause a dose-dependent increase in p65 NF-κB protein level in both fractions, but it was significantly higher in the nuclear fraction. This effect of ET-1 has previously been attributed to the enhanced translocation of p65 protein from the cytoplasm to the nucleus, and demonstrates NF-κB activity. Gerstung et al. [11] indicated that the action of ET-1 results in activation of the NF-κB pathway by the ET receptor in human renal tubular epithelial cells. Also previous experiments carried out on animals provide evidence that increased activation of NF-κB increases the expression of ET-1 in the pulmonary arteries [31], blood, liver [20], and kidney [28]. In the kidney, overexpression of ET-1 has been implicated in the pathogenesis of many kidney diseases, suggesting a role in kidney injury [1,34].

BAY 11-7082 selectively inhibits the phosphorylation of NF-κB, reducing nuclear factor translocation of NF-κB.

**References**


The authors have no potential conflicts of interest to declare.