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Inhibitor of NF-κB pathway (JSH - 23) inhibit oxidative stress generated by endothelin-1(ET-1)

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Abstract*— *ET-1 is an endogenous, vasoconstrictive peptide which can induce overproduction of reactive oxygen species and pro-inflammatory cytokines. The purpose of this study was to investigate the effect of JSH-23 (inhibitor of NF-κB pathway) on ET-1 induced oxidative stress and inflammation, and whether this actions is associated with activation of Nrf2 pathway. Experiments were performed on rats. The animals were randomly divided into four groups:

Group 1 (control group): received two doses of 0.2 ml saline, 0.5 h apart. Group 2 (saline + ET-1 group; served as ET-1 control): the rats were given 0.2 ml saline and ET-1 (8 µg/kg) 0.5 h later. Group 3 (saline + JSH-23): rats received 0.2 ml of JSH-23 (2mg/kg) and 30 min later 0.2 ml of saline. Group 4 (JSH-23+ET-1): rats received a single dose of JSH-23 (2 mg/kg) and a single dose of ET-1 (8 µg/kg) after 0.5 h.

ET-1 administration caused increase in TNF–α level and expression of subunit p65 NF-κB and decrease in SOD-1 level and Nrf2 expression. However, JSH-23 inhibited ET-1- induced increased in TNF-α, expression of subunit NF-κB p65 and enhanced level of SOD-1 and expression of Nrf2.

JSH-23 inhibited oxidative stress and inflammation by inhibited NF-κB pathway, activation pathway of Nrf2 and increase in SOD-1level.

Keywords*— *endothelin-1 (ET-1), inhibitor of NF-κB pathway (JSH - 23), oxidative stress, inflammation.

I. Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide synthetized predominantly in vascular endothelial cells from preproET-1 which is then converted to big - ET-1 and active ET-1 by proteases [1].

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ET-1 actions by binding to at least 2 endothelin receptors: ET_A and ET_B belonging to the superfamily of G-protein coupled receptors.

The ET_A receptor is found predominantly in smooth muscle cells and cardiac muscles [1] where mediate contraction [2], whereas the ETB receptor is abundantly expressed in endothelial cells [1] and stimulate the production of the vasodilators prostacyclin and nitric oxide (NO) [3]. Activation of the ET receptor type A and ET receptor type B promotes oxidative stress and a compromised free radical scavenging ability [4]. In turn, an increased ROS stimulates the mitogen activated protein kinase (MAPK) pathway and promotes upregulation of ET receptors [5]. It is well known that oxidative stress is generated via ROS that plays an important role in dysfunction skeletal muscle [6]. Studies performed in adult models of muscle atrophy showed that muscle fibers injury is associated with oxidative stress and increased mitochondrial production of ROS play a key role in triggering the atrophic signals [7].

ROS have been reported as key mediators to induce ET-1 [8] in in vitro and in vivo conditions [9], [10] and in turn ET-1 plays an important role to increase ROS level in the skeletal muscle [11]. Increased levels of ET-1 reduces blood flow in skeletal muscle [12] and inhibition ET_A receptor improve blood flow to active skeletal muscle [13]. In addition, elevated ET-1 levels lead to NADPH oxidase activation and superoxide formation via ET_A receptors, resulting in excessive ROS production and endothelial dysfunction [14]. Other studies have demonstrated that the kinases (p38 MAPK, ERK1/2) and NF-κB signaling pathways take part in the regulation of ET-1 in response to a variety of stimuli [15].

Nuclear factor-kappa B (NF- κB) is a sequence-specific transcription factor that plays a critical role in regulating the inducible gene expression in immune and inflammatory responses [16]. In resting cells, NF-κB is located in the cytoplasm with its inhibitory subunit IκBα. ROS stimulation result in serine phosphorylation of IκBα, which triggers its proteasomal degradation and subsequently activates NF-κB. NF-κB after activation translocates to the nucleus and induces the expression of specific gene product.

JSH-23 is regarded as an *in vivo* inhibitor of NF-κB and has been previously used as an antioxidant compound to counteract the toxic effect of free radicals and to interfere with the generation of proinflammatory cytokines in rats [17], [18].

Nrf2 is transcription factor which protect cells against oxidative stress. This factor controls expression of many cytoprotective enzymes such as: superoxide dismutase (SOD-1), heme oxigenase (HO-1), gamma-glutamyl cysteine synthetase (g-GCS), glutathione (GSH) [19], [20].

JSH-23 has been reported to have a protective effect against inflammation and oxidative stress in various organs. However, the effects of JSH-23 on ET-1 induced muscle injury yet was not examined. The purpose of this study was to investigate the effect of JSH-23 on ET-1 induced oxidative stress and whether this action is associated with activation of Nrf2 pathway.

II. Materials and methods

Animals. All experiments were carried out on male Wistar rats aged 2-3 month, weighing 200–230 g. The animals 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/£418/2008.

Experimental protocol. The rats were randomly assigned to fourth experimental groups as follows:

Group 1 (control group): received two doses of 0.2 ml saline, 0.5 h apart. Group 2 (saline + ET-1 group; served as ET-1 control): the rats were given 0.2 ml saline and ET-1 (8 µg/kg) 0.5 h later. Group 3 (saline + JSH-23): rats received 0.2 ml of JSH-23 (2mg/kg) and 30 min later 0.2 ml of saline. Group 4 (JSH-23+ET-1): rats received a single dose of JSH-23 (2 mg/kg) and a single dose of ET-1 (8 µg/kg) after 0.5 h.

The animals were anesthetized with urethane solution (60 mg per 100 g of body weight *ip.).* All drugs were administrated directly into the isolated femoral vein.

Tissue preparation and collection of samples. Five hours after the last injection of drugs, rats were sacrificed. Skeletal muscle was cut off of right thigh and rinsed with ice-cold saline, dried by blotting between two pieces of filter paper, weighed and frozen in -75^{o}C until used for measurements.

Skeletal muscle TNF-α, and SOD-1 assay. TNF-*a* and SOD-1 in the skeletal muscle were assayed by specific enzyme linked immunosorbent assay using a commercially-available ELISA test kit containing a monoclonal antibody specific for rat TNF-*a*, and SOD-1 (Cloud-Clone Corp, USA). Protein concentration of the supernatants was determined using Bradford method. Supernatant was removed and assayed immediately. Optical density of the supernatant (450 nm) was read using Victor x3 (Perkin Elmer, USA).The TNF-*a* and SOD-1 concentration were read from standard curves and expressed in pg/mg protein. All tests were performed in duplicate.

Total RNA extraction and cDNA generation. Total RNA was extracted from samples using RNeasy mini kits (Qiagen). RNA was quantified using PicoDrop spectrophotometer (Picodrop Limited, UK). The quality of RNA samples was analyzed by measuring the ratio of absorptions at 260/280 nm. The purified total RNA was immediately used for cDNA synthesis or stored at −80 ^{0}C. Generation of cDNA was performed with QuantiTect Reverse Transcription Kit (Qiagen) according to the protocol of the manufacturer. 1 µg of total RNA was used as starting material, reverse transcription was performed in conditions optimized for use with this kit (25°C for 10 min, 37°C for 120 min, 85°C for 5 min). The cDNA samples were kept frozen at −20°C.

Real Time PCR. mRNA quantification was done using standard TaqMan® Gene Expression Assays (Applied Biosystems): Nfe2l2 (Assay ID: Rn00477784_m1) and Actb (Rn00667869 m1) as a control. The 20 µl qPCR included 50 ng cDNA, 10 µl TaqMan Universal PCR Master Mix and 1 µl TaqMan Gene Expression Assay (20x). The reactions were incubated in a 96-well plate at 95^{0}C for 10 min, followed by 40 cycles of 95^{0}C for 15 s, and 60^{0}C for 1 min. All reactions were run in triplicate.

TaqMan PCR assays were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) and analyzed using Sequence Detection System 2.3 Software. Fold induction values (RQ) were calculated according to the equation 2-ΔΔCt, where ΔCt represents the differences in cycle threshold numbers between the target gene and endogenous control, and ΔΔCt represents the relative change in these differences between examined and control groups.

Statistical Analysis. The data are presented as mean ± S.E.M. if not stated otherwise, from 8 animals in each group. The statistical analysis was performed by ANOVA followed by the Duncan's multiple range test as post hoc. *P* values lower than 0.05 were considered significant.

III. Results

Effect of ET-1 and JSH-23 on TNF-α level. The level of TNF-α in skeletal muscle of saline-treated rats significantly lower than these ET-1 treated group ($p<0.001$). Administration of JSH-23 significantly improved TNF-α level as compared with ET-1 treated group ($p<0.01$). This decrease in proinflammatory cytokine probably reflects inhibition of NF-κB activation loop which up-regulates the production of TNF-α. (see of Fig. 1).

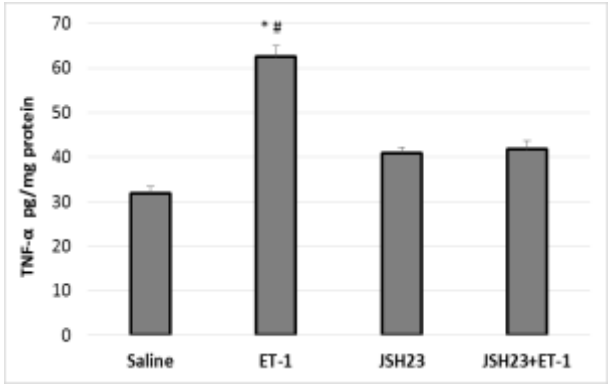

Figure 1. Effect of ET-1 and JSH-23 on TNF-α level. *p<0.01 vs saline, #p<0.001 vs JSH-23, JSH-23+ET-1.

Effect of ET-1 and JSH-23 on NF-κB/p65 expression. Administration of ET-1 caused increase in expression of NF-κB/p65 in skeletal muscle as compared to saline group ($p<0.05$). ET-1 mediated elevation of NF-κB subunit p65 was abrogated after pre-treatment with JSH-23, suggesting that JSH-23 modulates translocation of NF-κB subunit p65 ($p<0.05$). (see of Fig. 2).

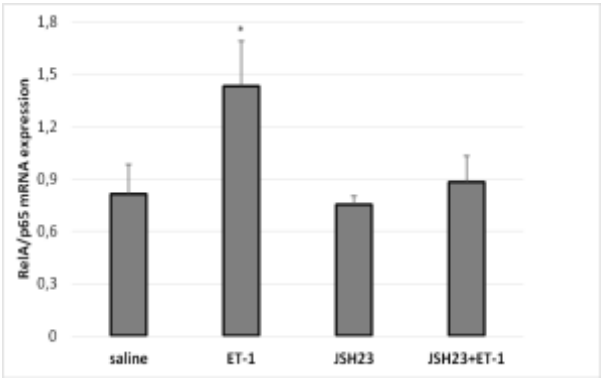

Figure 2. Effect of ET-1 and JSH-23 on NF- κB /p65 expression. *p<0.05 vs saline, JSH-23, JSH-23+ET-1.

Effect of ET-1 and JSH-23 on SOD-1 expression. In ET-1 group expression of SOD-1 was lower than the control group (p<0.01). SOD-1 expression was a significantly increased in the group JSH-23 +ET-1 as compared to ET-1 group (p<0.01). (see of Fig. 3).

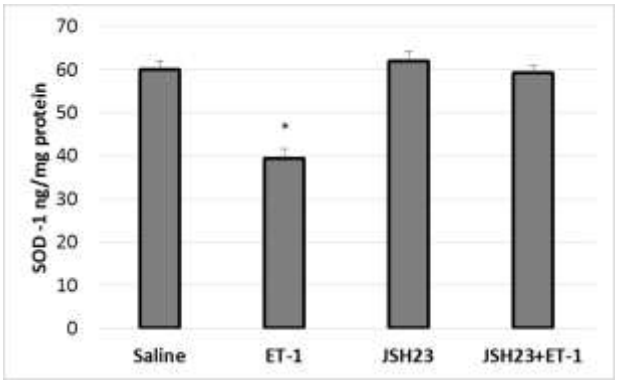

Figure 3. Effect of ET-1 and JSH-23 on SOD-1 expression. *p< 0.005 vs saline, JSH-23 and JSH-23+ET-1.

Effect of ET-1 and JSH-23 on Nrf2 expression. As shown in Fig. 4 treatment with ET-1 nonsignificantly decreased expression of Nrf2 as compared to the control group (p>0.05). Treatment with JSH-23 30 min before ET-1 administration inhibited ET-1 induced Nrf2 expression (p> 0.05).

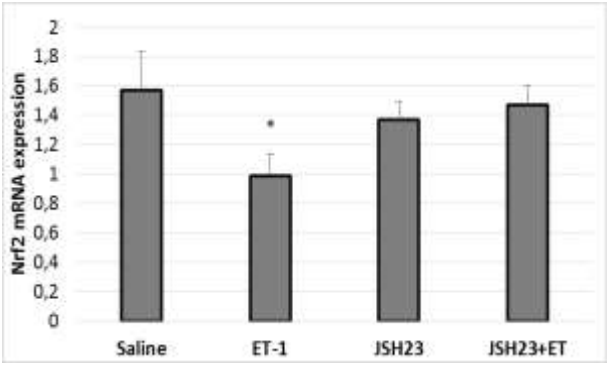

Figure 4. Effect of ET-1 and JSH-23 on Nrf2 expression . *p<0.05 vs JSH-23.

IV. Discussion

The main new findings of the study is that JSH-23 increased expression of Nrf2 in skeletal muscle in ET-1-treated rats. The increased expression of Nrf2 was associated with enhanced levels of SOD-1 and decreased production TNF-α in skeletal muscle.

ET-1 is potent vasoconstrictor and mitogen, possesses proinflammatory properties and is implicated in numerous cardiovascular diseases [21]. In addition, there is evidence that ET-1 increases superoxide generation in some cells by stimulating NADPH oxidase contributing to oxidative stress [22], smooth muscle cells [23].

In our study ET-1 enhanced TNF-α level in skeletal muscle homogenates. It was previously shown that ET-1 stimulates tissue cell to TNF-α release [24], [25], which *in turn* increases the generation ROS in various cell types *via* signaling with nuclear factor κB (NF-κB) and NADPH oxidase [26]. In our study increased TNF-α level was accompanied with enhanced expression of RelA/p65 mRNA. This is consistent with other reports which suggested that free radicals can activate NF-κB pathway [27].

In our studies administration of JSH-23 caused decrease in TNF-α level and decrease in expression p65 subunit mRNA, which testifies to antiinflammatory effect of this compound. Other authors also observed decrease in concentration of TNF-α level after JSH-23 administration [28], [29].

The elevated level of reactive oxygen species (ROS) increases protein degradation, reduces protein synthesis and damage of DNA leading to skeletal muscle decay. Moreover, TNF-α stimulates also the production of ET-1 [30] and other cytokines that acting together leads to developed inflammation and oxidative stress. Oxidative stress occurs when the production of ROS exceed the ability of antioxidants to scavenge and neutralize them. Superoxide radical, generated primarily in mitochondria as a consequence of normal metabolic processes, is the primary oxygen radical produced in the cell, and its production may be increased in pathological conditions [31].

The cells has a number of protective mechanisms to prevent to toxicity from superoxide [32] including SOD-1, HO-1, CAT, GSH-Px and the nonenzymatic free radical scavengers.

SOD-1 is a potent protective enzyme the first line against ROS. It can selectively scavenge O_2^- by catalysing its dismutation to H_2O_2 and oxygen (O_2). Then the other antioxidant, catalase (CAT) catylyzes the conversion of H_2O_2 to water and oxygen. Beside, SOD-1 is enzyme of phase II mediated by Nrf2 that serves as a defense mechanism against oxidative damage [33]. Our results presented here showed a non-significant decrease in SOD-1 level in ET-1 treated animals, which indicates the development of oxidative stress. Likewise, Mustaq et al. reported decrease in SOD-1 activity in heart muscle after ET-1 administration [34]. Also Tong et al. observed increase in ET-1level and decrease in SOD-1 levels in oxidative stress induced by ischemia-reperfusion in skeletal muscle [35]. Similarly, Ramiro-Diaz et al. observed enhanced level of ET-1 and decrease of SOD-1 activity in pulmonary arteries during hypoxia [23].

In our study pretreatment rats with JSH-23 resulted in enhanced SOD-1 levels, which pointing to the increasing strength of antioxidant defense. Also other inhibitor of NF-κB pathway (BAY 11-7082) increased SOD-1 level in diabetic rats [36].

ET-1 infusion resulted in increase in gene expression of Nrf2 in skeletal muscle which seems to be a countervailing mechanism to protect tissue form oxidative injury. This increase in the Nrf2 expression is consistent with increased oxidative stress in this experimental model. Under basal nonactivated conditions, Nrf2 interacts with cytosolic repressor protein Keap-1 (Kelch-like erythroid cell-derived protein 1). Upon activation by oxidative stress Nrf2 translocates to the nucleus where it binds to the ARE, triggering the transcription of antioxidant defense enzymes such as SOD-1 and HO-1, thus protecting tissues from oxidative injury indicated increased ET-1 mRNA level in liver and kidney in mice during oxidative stress induced by oxidized casein [38]. Likewise, Gomez-Guzman et al. observed increased plasma ET-1 level and enhanced expression of Nrf2 mRNA in aortic rings in DOCA (deoxycorticosterone acetate) - salts rats [37].

Our data also showed that JSH-23 administered alone or with ET-1 resulted in enhanced Nrf2 gene expression when compared to the control. Such effect of JSH-23 may protect skeletal muscle from ET-1 induced oxidative stress. Nrf2-ARE pathway coordinately up-regulates many protective detoxification and antioxidant genes, which can synergistically increase the efficiency of cellular defense system [39]. Moreover, increase in Nrf2 expression was associated with an increase in SOD-1 level. Nuclear factor-kappa B (NF-κB) is a ubiquitous transcription factor, that is known to regulate the expression of various genes whose early response products are crucial in the development of inflammation [16]. This factor is considered an oxidative stress-responsive element, which upon activation, translocates to the nucleus and induces the expression of specific gene products. In our study infusion of ET-1 resulted in an increase in gene expression of p65 subunit, which can indicates on develop of oxidative stress. Our previous study indicated that administration of ET-1 resulted in a development of oxidative stress in liver [25] and lungs [40] accompanied by a change in gene expression of p65 protein of NF-κB pathway.

Our results demonstrated that JSH-23 efficiently suppressed ET-1 induced NF-κB activation thus indicating a reduction in ROS production in skeletal muscle. Similarly, Ozkok et al. showed that JSH-23 administration caused inhibition of NF-κB activity in cisplatine-induced inflammation in kidney [28]. Kumar et al. indicated that JSH-23 inhibited nuclear translocation NF-κB in diabetic rats [18].

In this study we show that ROS generated by ET-1 enhanced p65 protein expression in skeletal muscle whereas JSH-23 (aromatic diamine) decreased p65 mRNA. The inhibition of nuclear translocation of NF-κB with JSH-23 caused an increase in Nrf2 expression as well as SOD-1 levels.

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