The International Journal of Biochemistry & Cell Biology 44 (2012) 311–319

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

A butyrolactone derivative suppressed lipopolysaccharide-induced autophagic injury through inhibiting the autoregulatory loop of p8 and p53 in vascular endothelial cells

Ning Meng[a,b], Jing Zhao[a,c], Le Su[a,c], BaoXiang Zhao[d,*], Yun Zhang[c], ShangLi Zhang[a,c], JunYing Miao[a,c,**]

[a] Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Science, Shandong University, Jinan 250100, China
[b] School of Medicine and Life Sciences, University of Jinan, Jinan 250022, China
[c] The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Health, Shandong University Qilu Hospital, Jinan, 250012, China
[d] Institute of Organic Chemistry, School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, China

ARTICLE INFO

Article history:
Received 1 August 2011
Received in revised form 30 October 2011
Accepted 2 November 2011
Available online 10 November 2011

Keywords:
Butyrolactone derivative
P8
P53
Autophagy
Lipopolysaccharide
Vascular endothelial cells

ABSTRACT

Lipopolysaccharide (LPS)-induced vascular endothelial cell (VEC) dysfunction is an important contributing factor in vascular diseases. Recently, we found that LPS impaired VEC by inducing autophagy. Our previous researches showed that a butyrolactone derivative, 3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO) selectively protected VEC function. The objective of the present study is to investigate whether and how 3BDO inhibits LPS-induced VEC autophagic injury. Our results showed that LPS induced autophagy and led to increase of reactive oxygen species (ROS) and decrease of mitochondrial membrane potential (MMP) in Human umbilical vein vascular endothelial cells (HUVECs). Furthermore, LPS significantly increased p8 and p53 protein levels and the nuclear translocation of p53. All of these effects of LPS on HUVECs were strongly inhibited by 3BDO. Importantly, the ROS scavenger N-acetylcysteine (NAC) could inhibited LPS-induced autophagy and knockdown of p8 by RNA interference inhibited the autophagy, p53 protein level increase, the translocation of p53 into nuclei and the ROS level increase induced by LPS in HUVECs. The data suggested that 3BDO inhibited LPS-induced autophagy in HUVECs through inhibiting the ROS overproduction, the increase of p8 and p53 expression and the nuclear translocation of p53. Our findings provide a potential tool for understanding the mechanism underlying LPS-induced autophagy in HUVECs and open the door to a novel therapeutic drug for LPS-induced vascular diseases.

1. Introduction

Lipopolysaccharide (LPS) is the biologically active constituent of endotoxin derived from the outer membrane of Gram-negative bacteria. It is recognized as the most potent microbial mediator implicated in the pathogenesis of sepsis and septic shock. LPS has been recently recognized as a potential mediator of inflammatory responses in atherosclerosis (Stoll et al., 2004). The host response to LPS is sufficient to generate diffuse endothelial injury. Many attempts to block LPS activity in clinical trials with septic patients have met with inconsistent and largely negative results. The hypothesis of LPS-driven inflammatory processes remains very attractive. However, few therapeutic options have been developed to date (Buttenschoen et al., 2010). Attractively, the groundbreaking discoveries into the precise molecular basis for LPS-mediated cellular activation and tissue injury have rekindled optimism that a new generation of therapies specifically disrupting LPS signaling might succeed (Opal, 2010). Autophagy, an evolutionarily

Abbreviations: 3BDO, 3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one; VEC, vascular endothelial cell; LPS, lipopolysaccharide; HUVECs, human umbilical vein endothelial cells; ROS, reactive oxygen species; MMP, mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; DCHF, 2′,7′-dichlorodihydrofluorescein; NAC, N-acetylcysteine; RNAi, RNA interference; siRNA, small interfering RNA; LC3-I, light chain 3-I; LC3-II, light chain 3-II; FBS, fetal bovine serum; FGF-2, fibroblast growth factor-2; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; OD, optical density; BSA, bovine serum albumin; PVDF, polyvinylidene fluoride.

* Corresponding author. Tel.: +86 531 88364929; fax: +86 531 88565610.
** Corresponding author at: Institute of Developmental Biology, School of Life Science, Shandong University, Jinan 250100, China. Tel.: +86 531 88364929; fax: +86 531 88565610.
E-mail addresses: bxzhao@sdu.edu.cn (B. Zhao), miaojy@sdu.edu.cn (J. Miao).

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doi:10.1016/j.biocel.2011.11.001

conserved cellular process, facilitates the turnover of long-lived proteins and organelles (Deretic, 2010). A growing body of evidence indicates excessive induction of the autophagic process by environmental or intracellular stress has an important role in several types of cardiovascular disease by functioning as a death pathway (Martinet and De Meyer, 2009). A recent report also demonstrated that autophagy proteins regulated innate immune response (Nakahira et al., 2011). Recently, we found that LPS impaired VEC by inducing autophagy (Meng et al., 2010). This information is providing novel interventions in the continuing efforts to find new therapeutic strategies.

We previously synthesized a series of butyrolactone derivatives, and found 3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO) could inhibit HUVEC apoptosis and senescence induced by deprivation of serum and FGF-2 (Wang et al., 2007). Our recent study showed that 3BDO protected VECs through selectively affecting Na^+, K^+-ATPase activity and mitochondria membrane potential during *in vitro* angiogenesis (Meng et al., 2008). Our further study showed that 3BDO could inhibit the accumulation of autophagic vesicles induced by chloroquine (CQ) in HUVECs (Huang et al., 2009). These findings suggest that 3BDO has a protective effect on VECs. Therefore, in this study we plan to investigate whether 3BDO inhibits LPS-induced VEC autophagic injury.

LPS treatment has previously been demonstrated to increase reactive oxygen species in endothelial cells (Park et al., 2004). Recent reports also highlighted the role of mitochondrial ROS on autophagy (Scherz-Shouval and Elazar, 2007). However, to date, no study has reported the role of ROS in LPS-induced VEC autophagy. In this study, we examined the effect of 3BDO on LPS-induced ROS production and determined whether ROS is involved in LPS-induced HUVEC autophagy.

p8 (nuclear protein 1, NUPR1; candidate of metastasis 1, com-1) is a protein related to the high mobility group of transcriptional regulators. It is a key player of the cellular stress response and is involved in several physiological and pathological processes (Goruppi and Iovanna, 2010). Accumulating evidence indicates that the transcriptional regulator p8 is also a key factor involved in autophagy and the role of p8 in autophagy is cell-type-specific (Salazar et al., 2009; Kong et al., 2010a,b). However, no report exists of the role of p8 in VEC autophagy. So, it is interesting to understand the function of p8 in LPS-induced VEC autophagy and investigate whether 3BDO can modulate p8 in this process.

It is known that p53 regulates autophagy in a dual fashion, depending on its subcellular localization, in nuclei, p53 can induce autophagy through transcriptional effects; in cytoplasm, it may act as a master repressor of autophagy (Zong and Moll, 2008; Maiuri et al., 2009). Our recent study showed that LPS promoted the nuclear translocation of p53 when it induced HUVEC autophagy (Meng et al., 2010). More interestingly, a previous report showed that there was an autoregulatory loop between the expressions of p8 and p53 in mouse embryonic fibroblasts (Vasseur et al., 2002), and a recent report showed that p8 could formed a complex with p53, transcriptionally regulates p21 and rescues breast epithelial cells from doxorubicin-induced genotoxic stress (Clark et al., 2008). However, the relationship between p8 and p53 in LPS-induced VEC autophagy and whether 3BDO can modulate the autoregulatory loop are not known. In this study we try to address these questions.

2. Materials and methods

2.1. Reagents, chemicals and preparation of drugs

Fetal bovine serum (FBS) and M199 medium were obtained from Hycolon Co. (Logan, UT). The antibodies specific to, β-actin, p8 and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against p62 was from BD Transduction Laboratories (Franklin Lakes, NJ). Antibody against LC3B was from Cell Signaling Technology (Danvers, MA). JC-1 was purchased from Invitrogen (Carlsbad, CA). NAC and DCHF were from Sigma–Aldrich (St. Louis, MO). LPS (*Escherichia coli* 055:B5) from Sigma–Aldrich (St. Louis, MO) was dissolved in distilled water as a stock solution at a concentration of 1 mg/mL. 3BDO was dissolved in dimethyl sulfoxide (DMSO) as a stock solution at a concentration of 200 mM. The final concentration of DMSO used in culture medium was below 0.1% (v/v) and did not affect cell viability.

2.2. Cell culture and treatment

HUVECs were obtained in our laboratory as described (Jaffe et al., 1973). HUVECs were cultured in M199 medium supplemented with 20% (v/v) fetal bovine serum and 10 IU/mL FGF-2 in a humidified incubator at 37.8 °C with 5% CO_2 and used for experiments at not more than passage 10. When HUVECs were grown to 80% confluency, the cells were stimulated with LPS. 3BDO treatments were performed 30 min prior cell exposure to LPS.

2.3. Western blot analysis

Cells under various treatments were lysed in lysis buffer containing 25 mM Tris–HCl (pH 6.8), 2% SDS, 6% glycerol, 1% 2-mercaptoethanol, 2 mM PMSF, 0.2% bromophenol blue and a protease inhibitor cocktail for 10 min at room temperature and boiled for another 10 min. The protein concentration was determined by Coomassie brilliant blue protein assay. Equal amounts of total proteins (20 μg) underwent 15% SDS–PAGE and were electroblotted onto polyvinylidene difluoride membrane. The membrane was blocked with 5% (w/v) nonfat dry milk in PBS–Tween 20 (PBST; 0.05%) for 1 h and incubated with anti-LC3, anti-p62, anti-p8, anti-p53 or anti-β-actin antibodies at 4 °C overnight. After a washing in TBST and TBS, the membrane was incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive bands were chromogenously developed with 3,3′-diaminobenzidine. β-Actin was used as a loading control. The relative quantity of proteins was analyzed by use of Quantity One software (Bio-Rad, Hercules, CA). LC3-II levels were measured relative to the loading control (β-actin). Densitometric analysis of LC3-II levels, from experiments performed at least in triplicate, was shown relative to level of β-actin.

2.4. Immunofluorescence microscopy

After treatment, cells were fixed with 4% paraformaldehyde and blocked with 3% normal goat serum for 15 min at room temperature. Cells were incubated with p53 or LC3 primary antibody (1/100) at 4 °C overnight. After three rinses in 0.1 M phosphate-buffered saline, cells were treated with a corresponding FITC-conjugated secondary antibody (1/200) in a humid chamber at 37 °C for 1 h. Cells were rinsed three times with 0.1 M PBS to eliminate the uncombined secondary antibody. A laser scanning confocal microscope (Leica, Wiesbaden, Germany) was used for fluorescence detection. Images are representative of three independent experiments.

2.5. Mitochondrial membrane potential (MMP) measurement

Mitochondrial membrane potential was estimated by fluorescence of JC-1 aggregates that are formed as a function of inner mitochondrial membrane potential (Smiley et al., 1991; Schulz et al., 2008). The formation of JC-1 aggregates and their fluorescence responds linearly to an increase in membrane potential (Smiley et al., 1991). After treatment, the cells plated on 24-well plates were