Toxicology and Applied Pharmacology 288 (2015) 374–386

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Anti-inflammatory effects of methylthiouracil in vitro and in vivo

Sae-Kwang Ku [a], Moon-Chang Baek [b,*], Jong-Sup Bae [c,**]

[a] Department of Anatomy and Histology, College of Korean Medicine, Daegu Haany University, Gyeongsan 712-715, Republic of Korea
[b] Department of Molecular Medicine, CMRI, School of Medicine, Kyungpook National University, Daegu 700-422, Republic of Korea
[c] College of Pharmacy, CMRI, Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea

ARTICLE INFO

Article history:
Received 23 June 2015
Revised 25 July 2015
Accepted 14 August 2015
Available online 20 August 2015

Keywords:
Drug repositioning
Methylthiouracil
Lipopolysaccharide
Barrier integrity

ABSTRACT

The screening of bioactive compound libraries can be an effective approach for repositioning FDA-approved drugs or discovering new treatments for human diseases. Here, methylthiouracil (MTU), an antithyroid drug, was examined for its effects on lipopolysaccharide (LPS)-mediated vascular inflammatory responses. The anti-inflammatory activities of MTU were determined by measuring permeability, human neutrophil adhesion and migration, and activation of pro-inflammatory proteins in LPS-activated human umbilical vein endothelial cells and mice. We found that post-treatment with MTU inhibited LPS-induced barrier disruption, expression of cell adhesion molecules (CAMs), and adhesion/transendothelial migration of human neutrophils to human endothelial cells. MTU induced potent inhibition of LPS-induced endothelial cell protein C receptor (EPCR) shedding. It also suppressed LPS-induced hyperpermeability and neutrophil migration in vivo. Furthermore, MTU suppressed the production of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, and the activation of nuclear factor-κB (NF-κB) and extracellular regulated kinases (ERK) 1/2 by LPS. Moreover, post-treatment with MTU resulted in reduced LPS-induced lethal endotoxemia. These results suggest that MTU exerts anti-inflammatory effects by inhibiting hyperpermeability, expression of CAMs, and adhesion and migration of leukocytes, thereby endorsing its usefulness as a therapy for vascular inflammatory diseases.

Introduction

Human vascular endothelial cells respond to proinflammatory stimuli by expressing adhesion and signaling molecules for white blood cells (Zimmerman et al., 1996). Organized and regulated expression of adhesion and signaling factors by human endothelial cells is an important feature in the ordered regulation of inflammatory responses, but dysregulated adhesion or signaling of endothelial cells is a pivotal mechanism of inflammatory injury (Zimmerman et al., 1996; Ross, 1999). Therefore, injury of endothelial cells is a critical phenomenon in the development of vascular inflammation. These cells act as a pivotal membrane barrier by protecting against leukocyte adhesion and transendothelial migration (TEM), thus maintaining vascular integrity (Mosnier et al., 2007). Therefore, maintaining vascular endothelial integrity is important for anti-inflammatory responses against inflammatory stimuli (Mosnier et al., 2007).

Endothelial protein C receptor (EPCR) is a type I transmembrane protein with homology to CD1/MHC class I proteins, most members of which are involved in inflammation (Esmon, 2006). EPCR has high binding affinity toward protein C and activated protein C (APC), and it accelerates the formation of APC, a potent anticoagulant and anti-inflammatory agent (Fukudome and Esmon, 1994; Stearns-Kurosawa et al., 1996). The baseline plasma concentration of EPCR is approximately 100 ng/mL (Kurosawa et al., 1997). The level can increase by at least five fold in inflammatory diseases (Kurosawa et al., 1998). In vitro studies have demonstrated that a wide variety of inflammatory mediators (IL-1β, H_2O_2, phorbol-12-myristate 13-acetate) and thrombin can cause a dramatic increase in EPCR shedding from the endothelium, and that EPCR shedding is potentiated by the microtubule disrupting agent, nocodazole (Xu et al., 2000). Soluble EPCR (sEPCR) is almost identical in size to full-length EPCR and retains its affinity for protein C and APC (Xu et al., 2000). sEPCR inhibits activation of protein C by competing with the membrane form of EPCR on the vessel wall, and it inhibits APC anticoagulant activity by blocking the interaction of APC with negatively charged membrane surfaces, an interaction that is necessary for efficient inactivation of factors Va and VIIIa (Kurosawa et al., 1997; Liaw et al., 2000). sEPCR levels are increased in patients with systemic inflammatory diseases (Kurosawa et al., 1998).

Metalloproteinase-mediated ectodomain shedding has been reported to occur in many cellular receptors (Moss and Lambert, 2002). Tumor necrosis factor-α converting enzyme (TACE) is an important member of the ADAM (a disintegrin and metalloproteinase) family (Blobel, 2005). TACE and closely related matrix metalloproteinases

* Correspondence to: M.-C. Baek, Department of Molecular Medicine, School of Medicine, Kyungpook National University, 101 Dongin-dong 2 Ga, Jung-gu, Daegu 700-422, Republic of Korea.
** Correspondence to: J.-S. Bae, College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University, 80 Daehak-ro, Buk-gu, Daegu 702-701, Republic of Korea.
E-mail addresses: mcbaek@knu.ac.kr (M.-C. Baek), baejs@knu.ac.kr (J.-S. Bae).

http://dx.doi.org/10.1016/j.taap.2015.08.009
0041-008X/

work together as sheddases to cleave hundreds of diverse transmembrane substrates, including TNF-α (Black et al., 1997), transforming growth factor-α, L-selectin (Lee et al., 2003), β-amyloid precursor protein (Buxbaum et al., 1998), and growth hormone receptor (Zhang et al., 2000). Unfortunately, little is known regarding which inhibitors modulate the activity or expression of sheddase, and how such a broad palette of proteolytic activity integrates to modulate behaviors. Furthermore, therapeutics have targeted sheddases and their substrates, yet many of these inhibitors have failed in clinical trials (Fingleton, 2008). Therefore, a need exists to find new inhibitors of TACE-mediated degradation, which integrates multiple layers of signaling networks to coordinately influence cell behavior in various vascular inflammatory diseases.

Discoveries about the molecular basis of disease provide unprecedented opportunities to translate research findings into new medicines. However, developing a brand-new drug takes an enormous amount of time, money, and effort, mainly because of bottlenecks in the therapeutic development process (Dickson and Gagnon, 2004). "Repositioning" generally refers to studying a compound or biologic (referred to as agents) used to treat one disease or condition to see if it is safe and effective for treating other diseases (O'Connor and Roth, 2005; Padhy and Gupta, 2011; Liu et al., 2013). Many agents approved for other uses have already been tested in humans, so detailed information about their pharmacology, formulation, and potential toxicity is available. Because repositioning builds upon previous research and development efforts, new candidate therapies could be ready for clinical trials quickly, speeding their review by the FDA and, if approved, integration into health care (O'Connor and Roth, 2005; Padhy and Gupta, 2011; Liu et al., 2013). In our search for repositioned FDA-approved drugs (total of 1163), 327 drugs were selected which are related to vascular inflammation and infection. Among the selected drugs, a high content screening system (PerkinElmer Operetta, Waltham, MA) was used to select the compounds that modulate LPS-mediated vascular endothelium disruption, and we found that methylthiouracil (MTU, antithyroid drug) had anti-inflammatory effects on LPS-mediated severe inflammatory responses. Here, we report the barrier protective effects of MTU on LPS-mediated vascular barrier disruption in vitro and in vivo.

Materials and methods

Reagents

Methylthiouracil (MTU) was purchased from Abcam (Cambridge, MA). Bacterial LPS (serotype: 0111:B4, L5293), Evans blue, crystal violet, 2-mercaptoethanol, polyethylene-glycolated (PEG)-catalase, and antibiotics (penicillin G and streptomycin) were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and Vybrant DiD were purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal TLR4 antibody (H-80, sc-1074) was purchased from Santa Cruz Biotechnology (Dallas, TX).

Animals and husbandry

Male C57BL/6 mice (6–7 weeks old; average weight, 27 g) purchased from Orient Bio Co. (Sungnam, Republic of Korea) were used in this study after a 12-day acclimatization period. The animals were housed 5 per polycarbonate cage under controlled temperature (20–25 °C) and humidity (40–45% RH) and a 12:12-h light/dark cycle. Animals received a normal rodent pellet diet and water ad libitum during the acclimatization. All the animals were treated in accordance with the 'Guidelines for the Care and Use of Laboratory Animals' issued by Kyungpook National University (IRB No; KNU 2014-13).

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA) and maintained as previously described (Ku et al., 2013a, 2014; Lee et al., 2013, 2015b; Bae et al., 2014; Ku and Bae, 2014a,b; Yoo et al., 2014). Briefly, the cells were cultured to confluency at 37 °C and 5% CO_2 in endothelial basal medium (EBM)-2 basal media supplemented with growth supplements (Cambrex Bio Science). Human neutrophils were freshly isolated from whole blood (15 mL) obtained by venipuncture from five healthy volunteers and maintained as previously described (Hofbauer et al., 1998; Bae and Rezaie, 2013).

Permeability assay in vitro

For spectrophotometric quantification of endothelial cell permeabilities in response to increasing concentrations of MTU for 6 h, the flux of Evans blue-bound albumin across functional cell monolayers was measured using a modified 2-compartment chamber model, as described previously (Bae and Rezaie, 2011).

In vivo permeability assay

Mice were administered LPS (0.3 mg/mouse or 15 mg/kg, intravenously). After 4 h, the mice were intravenously treated with MTU (142 or 284 μg/kg, for 6 h) and injected with 1% Evans blue dye solution in normal saline. Six hours later, the mice were sacrificed and peritoneal exudates were collected by washing cavities with 5 mL of normal saline and by centrifuging at 200 ×*g* for 10 min. The absorbance of the supernatant was read at 650 nm. Vascular permeabilities are expressed as μg of dye/mouse that leaked into the peritoneal cavity and were determined using a standard curve, as previously described (Lee et al., 2009; Bae et al., 2012).

ELISA for TLR4

The expression of toll like receptor 4 (TLR4) on HUVECs was determined by whole-cell enzyme-linked immunosorbent assay (ELISA), as described previously (Kim et al., 2011).

ELISA for phospho p-38

Measurement of phospho p-38 expression was performed according to the manufacturer's instructions using a commercially available ELISA kit (Cell Signaling Technology, Danvers, MA).

Immunofluorescence staining

HUVECs were grown to confluence on glass cover slips coated with 0.05% poly-L-lysine in complete media containing 10% FBS and maintained for 48 h. The cells were then stimulated with LPS (100 ng/mL) for 4 h, with or without post-treatment with MTU (10 or 20 μM) for 6 h. For cytoskeletal staining, the cells were fixed in 4% formaldehyde in PBS (v/v) for 15 min at room temperature, and for immunostaining, the cells were permeabilized in 0.05% Triton X-100 in PBS for 15 min and blocked in the blocking buffer (5% bovine serum albumin in PBS) overnight at 4 °C. The cells were incubated with primary rabbit monoclonal NF-κB p65 antibody, anti-rabbit Alexa 488, and F-actin labeled fluorescein phalloidin (F 432; Molecular Probes, Invitrogen) overnight at 4 °C. Nuclei were counterstained with 4,6-diamidino-2-phenylindole phenylindole (DAPI), and the cells were visualized by confocal microscopy at 630× magnification (TCS-Sp5, Leica Microsystem, Germany). Quantification of p65 nuclear translocation was calculated, as described previously (Fuseler et al., 2006).

Cell viability assay

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) was used as an indicator of cell viability. Cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were