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LXR agonism improves TNF-α-induced endothelial dysfunction in the absence of its cholesterol-modulating effects

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ABSTRACT

Stimulation of the liver X receptor (LXR) is associated with anti-inflammatory and vascular-protective effects under hyperlipemic conditions. We examined whether LXR stimulation influences TNF-α-induced endothelial dysfunction under normolipemic conditions. Endothelium-dependent vasorelaxation of aortic rings was determined in an organ water bath. Human umbilical vein endothelial cells (HUVEC) were exposed to TNF-α (10 ng/ml) in the presence or absence of 5 μM of the LXR agonist T0901317 or GW3965 and changes in TNF-α-induced endothelial cell apoptosis, inflammation, oxidative stress, and NO metabolism were analyzed. T0901317 improved TNF-α-impaired endothelium-dependent relaxation of aortic rings in response to acetylcholine. T0901317 decreased the TNF-α-induced apoptosis and inflammation as indicated by a decrease in caspase 3/7 activity, VCAM-1 mRNA expression and subsequent mononuclear cell adhesion. Furthermore, T0901317 reduced the expression of the oxidative stress markers: AT1R, NOX4, and p22phox and normalized the TNF-α-induced NOX activity to basal levels. In line with the reduced AT1R expression, T0901317 impaired the Ang II responsiveness. T0901317 influenced NO metabolism as indicated by a decrease in TNF-α-upregulated arginase activity, a reversal of TNF-α-induced downregulation of *argininosuccinate synthase* mRNA expression and eNOS expression to basal levels and a raise in NO production. Furthermore, T0901317 decreased the TNF-α-induced superoxide and nitrotyrosine production, but did not upregulate the TNF-α-downregulated eNOS dimer/monomer ratio. Silencing of LXRβ, but not of LXRα, abrogated the anti-apoptotic effects of T0901317. We conclude that LXR agonism improves TNF-α-impaired endothelial function via its anti-apoptotic, anti-inflammatory, and anti-oxidative properties and its capacity to restore TNF-α-impaired NO bioavailability independent of its cholesterol-modulating effects.

1. Introduction

Vascular endothelial dysfunction occurs at the early onset of atherogenesis. It is characterized by a reduction of the bioavailability of the major endothelium-derived vasoactive mediator, nitric oxide (NO) [1] and takes place in the absence of any structural changes of the vessel wall. NO is produced by the action of (endothelial) NO synthase (eNOS) which converts L-arginine to L-citrulline. Among others, deficit of its substrate L-arginine, due to increased arginase activity [2] or impaired recycling of L-citrulline to L-arginine [3] results in impaired NO production.

Inflammation is an important trigger of endothelial dysfunction [4]. Among the cytokines, TNF-α is known to affect NO production by 1) reducing eNOS expression [5], 2) to increase the natural competitor of eNOS, arginase [2], and 3) to decrease the recycling of L-citrulline to L-arginine via the downregulation in argininosuccinate synthase expression [6]. Furthermore, TNF-α induces reactive oxygen species (ROS) [7], involving NAD(P)H oxidase (NOX) [8] and uncoupled eNOS [9]. Consequently, TNF-α impairs endothelium-dependent relaxation [10].

Abbreviations: HUVEC, human umbilical vein cells; LXR, Liver X receptor; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; NOX, NAD(P)H oxidase; $O_2\bullet$, superoxide; ROS, reactive oxygen species; RXR, retinoid X receptor; TNF, tumor necrosis factor.

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Liver X receptors (LXR) are ligand-activated transcription factors that form heterodimers with the retinoid X receptor (RXR) and belong to the nuclear hormone receptor superfamily. Oxysterols and oxidized cholesterol derivates are endogenous ligands of LXRs. LXRs regulate the expression of genes involved in lipid and glucose metabolism and reverse cholesterol transport [11]. They also raise high-density lipoproteins (HDL) [12], which are besides their important role in reverse cholesterol transport also known for their endothelial-protective effects [13–15]. In addition, synthetic LXR agonists, such as T0901317 have been shown to have anti-inflammatory [16], anti-oxidative [17], and anti-apoptotic [18] features. Given these properties, LXRs have been suggested as potential targets for therapeutic intervention in human cardiovascular disorders [19]. Recently, LXRs have been shown to reduce plaque formation and to improve vasomotor function in atherosclerotic apo $E^{-/-}$ mice [20]. However, an impact of LXR agonism on endothelial function independent of its cholesterol lowering capacity has not been demonstrated before. Therefore, the aim of the present study was to evaluate whether LXR agonism improves TNF-α-induced endothelial dysfunction in aortic rings, independent of its cholesterol lowering capacity. Underlying mechanisms were analyzed by supplementation of the LXR agonist T0901317 on TNF-α-stimulated human umbilical vein cells (HUVEC).

2. Methods

2.1. Vasorelaxation studies in isolated rat aortic rings

Thoracic aortae from anaesthesized male Wistar rats were rapidly excised, cleaned of connective tissue, and cut into rings 2–3 mm in length for organ-chamber experiments as described previously [21]. Before mounting on the organ-chamber, rings were cultured in the presence or absence of 25 pg/ml of TNF-α, with or without 5 μM T0901317 for 24 h in DMEM medium. Rings were mounted on platinum hooks in 10 ml jacketed organ baths containing modified Krebs–Henseleit solution (composition, in mmol/l: NaCl 144, KCl 5.9, $CaCl_2$ 1.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and D-glucose 11.1) and 1 μmol/l diclofenac. Tension was gradually adjusted to 2 g over 1 h. The solution in the bath was maintained at 37 °C with a gas mixture of 5% CO_2 and 95% O_2. Following equilibration and submaximal pre-contraction with phenylephrine (0.05 μmol/l), relaxation to increasing concentrations (10 nmol/l to 10 μmol/l) of the endothelium-dependent vasodilator acetylcholine was performed to obtain cumulative concentration–response curves. Maintenance of smooth-muscle integrity was confirmed by evaluation of endothelium-independent vasodilation to sodium nitroprusside (SNP, 0.1–10 nM). Vasorelaxation is expressed as percentage of pre-contraction with phenylephrine.

2.2. Cell culture

HUVEC (7,500 or 10,000 and 150,000 per 96-well and 6-well, respectively) were cultured in endothelial basal medium and endothelial supplements (Promocell). After 24 h (h) of culture, medium was changed and cells were stimulated with or without 10 ng/ml of TNF-α in the presence or absence of 5 μM of the LXR agonist T0901317, which activates both LXRα and LXRβ. The concentration of 5 μM of T0901317 was determined in an experiment showing that among the evaluated concentrations (200 nM, 1 μM, 5 μM, and 10 μM), the most pronounced reduction in TNF-α-induced oxidative stress in HUVEC was reached by 5 μM of T0901317 (see Supplemental Fig. 1), a concentration also used in experiments with vascular smooth cells [22] and aortic cells [23]. Besides experiments with T0901317, HUVEC were also supplemented with TNF-α in the presence or absence of the LXR agonist GW3965 or L-NAME.

2.3. Caspase 3/7 activity assay

4 h after TNF-α stimulation in the presence or absence of 5 μM of T0901317 or 5 μM of GW3965, respectively, caspase 3/7 activity was determined with a Caspase Glo 3/7 activity kit (Promega) according to the manufacturer's protocol. Luminescence was measured in a microplate-reading luminometer (Mithras LB 940, Berthold Technologies GmbH & Co KG, Germany).

2.4. Real-time PCR quantification

Thirty minutes (min) after TNF-α stimulation, cells were collected in a lysis buffer (Miltenyi Biotech, Bergisch Gladbach, Germany). Next, mRNA was isolated and cDNA prepared with the MultiMACS One-step cDNA synthesis Kit (Miltenyi Biotech) according to the manufacturer's protocol. To analyze *LXRα*, *LXRβ*, *RXRα*, *VCAM-1*, *AT1R*, *NOX4*, *p22phox*, *arginase II*, and *argininosuccinate synthase* mRNA expression, quantitative real-time PCR (Eppendorf Mastercycler epgradient realplex, Hamburg, Germany) was performed. mRNA expression were normalized to 18S or L32 and relatively expressed with the control group set as 1. Commercial human *NOX4*, *p22phox*, *arginase II*, *argininosuccinate synthase*, and *18S* primers (Applied Biosystems, Carlsbad, CA, USA) or self-designed human *LXRα*, *LXRβ*, *RXRα*, *VCAM-1* (see Table 1 in Supplement), *AT1R* [13], and *L32* [13] primers were used. Absolute mRNA expression levels of *LXRα*, *LXRβ*, and *RXRα* towards L32 were quantified via the use of plasmid standards containing the respective amplified PCR fragments, as described previously [15].

2.5. Adhesion assay

Thirty min after TNF-α and/or T0901317 supplementation, HUVEC (10,000/96-well) were stimulated with 0 or 5 μM Ang II. 4 h after the TNF-α and/or T0901317 stimulation, HUVEC were washed and 100,000 DiO-labeled mononuclear cells (MNCs) activated with phorbolmyristate acetate (50 ng/ml)/ionomycin (500 ng/ml) were added to HUVEC for 30 min. After three washes with PBS, absorbance was measured at 517 nM with a Berthold fluorometer (Mithras LB 940, Berthold Technologies GmbH & Co KG, Germany). Data depict the absorbance of bound MNCs to HUVEC minus the absorbance of HUVEC without MNC.

2.6. NADPH oxidase activity

As described previously [13], NOX activity of HUVEC was analyzed according to Griendling et al. [24], slightly modified. In brief, 1 h after TNF-α and/or T0901317 supplementation, cells were washed in ice-cold PBS. Then, cells were scraped from the plate in 1 ml of ice-cold PBS and centrifuged for 10 min at 4 °C at 10,000 rpm. The supernatant was discarded and the pellet resuspended in 90 μl of cell lysis buffer (Invitrogen). NOX activity was measured by lucigenin-enhanced chemiluminescent detection of superoxide (O_2•) with a luminometer (Mithras LB 940, Berthold Technologies GmbH & Co KG, Germany). The reaction buffer contained 1 mM EGTA, 150 mM sucrose, 500 μM lucigenin and 1 mM of NADPH (180 μl). The reaction was started by addition of 20 μl of protein homogenate. Luminescence was measured as the rate of photon counts per μg protein, after substraction of the counts obtained from a buffer blank.