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RESEARCH ARTICLE

Lysosomal destabilization activates the NLRP3 inflammasome in human umbilical vein endothelial cells (HUVECs)

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Abstract Inflammation is a crucial component in the pathogenesis of many vascular diseases, such as atherosclerosis and diabetes. Inflammasomes are intracellular signalling complexes whose activation promotes inflammation. Nucleotide-binding domain and Leucine-rich repeat Receptor containing a Pyrin domain 3 (NLRP3) is a pattern recognition receptor (PRR) forming the best-known inflammasome. Disturbances in NLRP3 have been associated with multiple diseases. The purpose of this study was to explore the lysosomal destabilization-related NLRP3 inflammasome signaling pathway in human endothelial cells. In order to prime and activate NLRP3, human umbilical vein cells (HUVECs) were exposed to TNF-α and the lysosomal destructive agent Leusine-Leusine-O-Methylesther (Leu-Leu-OMe), respectively. A caspase-1 inhibitor was used to block caspase-1's enzymatic function and an interleukin 1 receptor antagonist (IL-1RA) to prevent any possible secondary effects of IL-1β. Leu-Leu-OMe increased the expression of NLRP3, IL-1β, and IL-18 in HUVECs. Exposure to Leu-Leu-OMe significantly promoted the production of IL-6 and IL-8 in primed HUVECs; this effect was prevented by the pre-treatment of cells with an IL-1RA. Our results suggest that lysosomal destabilization activates the NLRP3 inflammasome pathway that promotes the production of IL-6 and IL-8 in an autocrine manner in HUVEC cells.

Keywords Diabetic retinopathy · Atherosclerosis · Inflammasome · Inflammation · Angiogenesis

Abbreviations

EC	Endothelial cell
HUVEC	Human umbilical vein endothelial cell
NLRP3	Nucleotide-binding domain and leucine-rich repeat receptor containing a pyrin domain 3
IL	Interleukin
IL-1RA	Interleukin 1 receptor antagonist
PRR	Pattern recognition receptor
Leu-Leu-OMe	Leusine-Leusine-O-Methylesther
MVEC	Microvascular endothelial cell
FBS	Fetal bovine serum
TNF-α	Tumor necrosis factor α

Introduction

Innate immunity and inflammation play a major role in many vascular diseases, such as diabetes and atherosclerosis, causing both an economic and clinical burden worldwide (Menu et al. 2011; Nathan 2002). The incidence, prevalence, and progression of these vascular diseases are increasing, mostly due to changes in nutritional habits and the increased prevalence of the metabolic syndrome. The vascular endothelium regulates homeostasis between blood and tissues by controlling the energy and metabolite transportation through the vessel wall (Aird 2007). Endothelial cells (ECs) are also key regulators

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and major targets of inflammatory reactions. These cells respond to signals originating from inflamed tissues by further expressing inflammation-related mediators and by controlling the relocation of leukocytes from the bloodstream to the sites of inflammation (Kauppinen et al. 2016).

Recognized risk factors of (cardio)vascular diseases, such as hyperlipidemia and hyperglycemia, predispose the endothelium directly to inflammation. It has recently become evident that the Nucleotide-binding domain and Leucine-rich repeat Receptor containing a Pyrin domain 3 (NLRP3) inflammasome is an important initiator of the pathological changes occurring in the vascular endothelium. In mouse microvascular endothelial cells (MVECs), palmitate-induced NLRP3 inflammasome activation resulted in the reduction of inter-endothelial tight junction proteins ZO-1/ZO-2 (Wang et al. 2016). High glucose levels also promoted NLRP3 inflammasome activation, tight junction disruption, and endothelial hyperpermeability in MVECs (Chen et al. 2016). Moreover, it has been shown previously in HUVECs that oscillatory shear flow can trigger NLRP3 inflammasome activation, contributing to the formation of atherosclerotic lesions (Xiao et al. 2013).

Due to the multitude of different types of activators and the indirect activation process, it is apparent that NLRP3 can be activated via several distinct mechanisms. For example, lysosomal destabilization is known to be one of the major mechanisms associated with NLRP3 activation (Jo et al. 2016). Lysosomes are key players in an intracellular catabolic process called autophagy; this can be triggered by different stressors e.g. by energy deprivation and oxidative stress (Kaarniranta et al. 2013). Autophagy recycles building blocks for anabolic processes, but it becomes disturbed if there is an excessive nutrient load (Webster et al. 2014). A decline in the efficiency of autophagy can result in the activation of the inflammasome and thereby promote the progression of many metabolic, age-related, and inflammatory diseases (Nixon 2013; Piippo et al. 2014; Salminen et al. 2012).

In the present study, we have examined the effect of lysosomal destabilization on inflammasome signaling in human endothelial cells. With respect to the central role of the vascular endothelium in inflammatory processes, it is important to clarify mechanisms at the molecular level to find ways of combatting the prolonged inflammation present in pathological conditions.

Materials and methods

Cell cultures and treatments

Human umbilical vein endothelial cells (HUVECs) were provided by Prof. Seppo Ylä-Herttuala, AIV Institute, Department of Molecular Medicine and Biotechnology, University of Eastern Finland, Kuopio, Finland. The HUVECs had been isolated from umbilical veins obtained from the maternity ward of Kuopio University Hospital, and cultivated as described previously (Jaffe et al. 1973). Cell isolation was approved of the Kuopio University Hospital Ethics Committee. The cells were grown on 10 mm^2 plastic wells coated with 10 μg/ml fibronectin (Sigma, St Louis, MO) and 0.05% gelatin in a humidified 10% CO_2 atmosphere at 37 °C in endothelial cell (EC) growth medium (EGM Bullet Kit; 0.1% human epidermal growth factor, 0.1% hydrocortisone, 0.1% gentamycin - amphotericin-B, 0.4% bovine brain extract, 2% FBS (fetal bovine serum; Lonza, CC-3124). The same medium with FBS reduced to 0.5% was used in the experiments. Confluent cell cultures were washed with PBS and exposed to tumor necrosis factor alpha (TNF-α, 2 ng/ml; R&D systems) in a humidified 10% CO_2 atmosphere at 37 °C for 5 h. Thereafter, in experiments with inhibitors, a caspase-1 inhibitor (Caspase-1 Inhibitor II, 20 μM; Calbiochem) or an IL-1 receptor antagonist (IL-1RA, 100 ng/ml; R&D Systems) were added for 1 h (IL-1RA) and 30 min (Caspase-1 inhibitor) before the 19 h incubation with L-leucyl-L-leucine methyl ester (Leu-Leu-OMe; Chem-Impex International, Wood Dale, I). Control cells were maintained in endothelial cell growth medium with 0.5% FBS and supplemented with DMSO (DMSO, D2650, Sigma) where appropriate.

Cytokine measurements

Medium samples were collected after the incubations, centrifuged at 16060 x g for 10 min, and stored at −70 °C until analyzed. The concentrations of pro-inflammatory cytokines were measured using commercial ELISA assays (IL-1β, IL-6, and IL-8 OptEIA™ sets from BD Pharmingen, human IL-18 Platinum ELISA from eBioscience, and NLRP3 ELISA from Cusabio) as previously (Piippo et al. 2014).

Statistical analysis

All statistical analyses were performed using the GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Pairwise comparisons between groups were made with the Mann-Whitney *U*-test. *P* values below 0.05 were considered statistically significant.

Results

Lysosomal destabilization activates the NLRP3 inflammasome in human umbilical vein endothelial cells

In order to simulate inflammasome activation, HUVECs were primed with TNF-α and then exposed to the lysosomal

destructive agent Leu-Leu-OMe. TNF-α priming significantly increased the expression of NLRP3 (62.2× compared to control), and Leu-Leu-OMe further induced its expression (247.0 and 4.0× compared to both untreated and TNF-α-primed cells, respectively; Fig. 1). TNF-α alone did not promote the release of inflammasome-related IL-1β or IL-18 cytokines (Fig. 2a, b) but the exposure of primed HUVECs to Leu-Leu-OMe significantly increased the secretion of IL-18 (Fig. 2). Concurrently, no elevated levels of IL-1β were detectable in the cell culture medium and the IL-1β concentration seemed simply to decline after the exposure to Leu-Leu-OMe (Fig. 2b). In order to verify the specificity of this phenomenon, the cells were treated with a caspase-1 inhibitor prior to the exposure to TNF-α and Leu-Leu-OMe. This significantly increased the IL-1β levels in the culture medium, restoring them to a similar level as the primed cells (Fig. 2b). When the level of IL-1β was assayed from cell lysates, Leu-Leu-OMe doubled the IL-1β levels (Fig. 2c) suggesting that the cytokine was located within the cell rather than freely dissolved in the medium. We have previously shown that our ELISA assay measures mature IL-1β instead of its immature pro-form (Piippo et al. 2014). The present data indicate that lysosomal destabilization by Leu-Leu-OMe activates the NLRP3 inflammasome in HUVECs, resulting in the release of IL-18 and IL-1β, the latter of which binds to its receptors in an autocrine manner and therefore is detectable only in cell lysates.

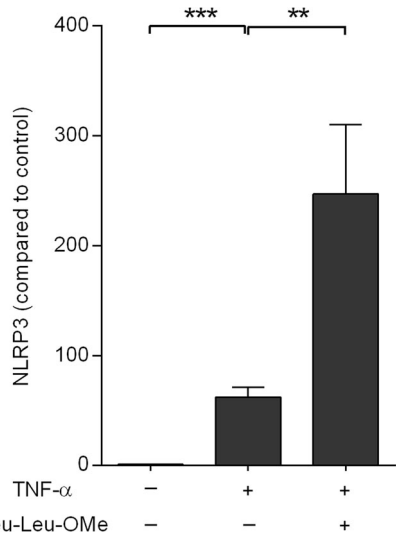

Fig. 1 Induction of NLRP3 by lysosomal destabilization in HUVECs. NLRP3 was measured from cell culture medium by the ELISA method. Three independent experiments, each with three parallel samples, were performed with values being normalized to the mean of control in the particular experiment. Combined data from three experiments are shown as mean ± SEM. ** denotes $P < 0.01$ and *** $P < 0.001$, Mann-Whitney *U*-test

Released IL-1β contributes to the production of IL-6 and IL-8

HUVECs can induce inflammation also via alternative pathways other than inflammasomes and therefore we measured IL-6 and IL-8 from the medium samples. TNF-α evoked 16.9 and 52.4 times higher concentrations of IL-6 and IL-8, respectively, when compared to untreated control cells (Fig. 3). Lysosomal destabilization with Leu-Leu-OMe further increased the cytokine release resulting in 103.5 and 100.7 times higher levels of IL-6 and IL-8, respectively, when compared to controls (Fig. 3). Those amounts were also 6.1 (IL-6) and 1.9 (IL-8) times higher than the cytokine levels released by TNF-α alone. Since it seemed possible that secreted IL-1β binds to its receptors on the cell surface, we blocked IL-1 receptors prior to the activating signal with an IL-1 receptor antagonist (IL-1RA). This significantly prevented the production of both IL-6 and IL-8, suggesting that IL-1β released by the inflammasome activation could further promote inflammation in the endothelium. The role of IL-1β is supported also by our data that the levels of IL-6 and IL-8 were significantly lower in the presence of a caspase-1 inhibitor.

Discussion

Dysregulated activation of NLRP3 inflammasome has been associated with the pathogenesis of many vascular disorders, most recently diabetic proliferative retinopathy (Duewell et al. 2010; Lee et al. 2013; Sandanger et al. 2013; Loukovaara et al. 2017). In the present study, we provided the priming signal for the inflammasome activation in HUVECs using TNF-α, which is a pro-inflammatory cytokine released rapidly upon the initiation of inflammation. TNF-α promotes the production of pro-IL-1β and NLRP3 in the cell by activating the NF-κB pathway (O'Connor et al. 2003). Our result showing significantly increased levels of NLRP3 after an exposure to TNF-α is in line with idea of inducible inflammasome receptor in HUVECs. The activation signal Leu-Leu-OMe recognized by NLRP3 then resulted in the assembly of the inflammasome complex with the subsequent release of mature cytokines. NLRP3 has multiple activation mechanisms; the three main ones are potassium efflux, lysosomal destabilization, and oxidative stress (Jo et al. 2016). Previously, palmitate-driven inflammasome activation in MVECs was prevented by treatment with a cathepsin B inhibitor (Wang et al. 2016), suggesting that lysosomal damage is capable of activating NLRP3 in endothelial cells. Furthermore, lysosomal membrane stabilizers also prevented NLRP3 inflammasome activation in a mouse model of coronary arteritis (Chen et al. 2015a). Our present results with the lysosomal destabilizer Leu-Leu-OMe are in line with these earlier findings. In previous studies, it has been shown that Leu-Leu-OMe activates NLRP3 inflammasomes in human

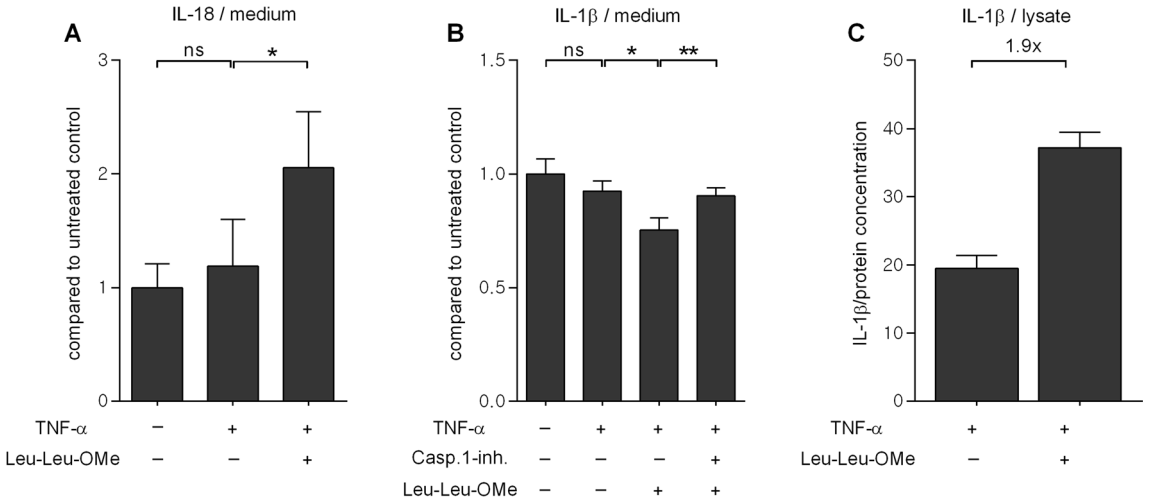

Fig. 2 Inflammasome-related cytokine production by Leu-Leu-OMe in HUVECs. The levels of IL-18 (**a**) and IL-1β (**b**) were measured from cell culture medium samples by the ELISA method after exposing cells to TNF-α for 5 h and then to Leu-Leu-Ome for 19 h, these agents served as priming and activation signals, respectively. The caspase-1 inhibitor (20 μM) was added prior to the activation signal where indicated. ELISA was also used to determine the cell-associated IL-1β concentration (**c**). Results are shown as mean ± SEM and are combined from 2 to 4 independent experiments with 2–6 parallel samples per group/experiment. * denotes $P < 0.05$, ** $P < 0.01$, and ns, not significant, Mann–Whitney *U*-test

retinal pigment epithelial (RPE) cells (Tseng et al. 2013), and it seems to act similarly also in HUVECs.

IL-1β is a strong pleiotropic pro-inflammatory cytokine, under stringent cellular regulation. In the endothelium, the functions of IL-1β include its pro-coagulant activity and the induction of adhesion molecules, which make it possible for the endothelium to trap leukocytes from the bloodstream (Bevilacqua et al. 1985). Previous studies have emphasized the crucial role of IL-1β in the disruption of tight junctions leading to an increase in vascular permeability (Chen et al. 2016; Chen et al. 2015a, 2015b; Wang et al. 2016). Inhibiting IL-1 activity has been shown to be effective in alleviating certain pathological conditions including type 2 diabetes and ischemic heart diseases (Abbate et al. 2010; Larsen et al. 2007; Van Tassell et al. 2012). Several IL-1 inhibitors have been demonstrated to be effective in treating cardiac diseases and diabetes. These inhibitors include anakinra (Kineret®; Swedish Orphan Biovitrum), a recombinant form of the naturally occurring IL-1 receptor antagonist (IL-1RA), which blocks the activity of both IL-1α and IL-1β; the dimeric fusion protein rilonacept (Arcalyst®; Regeneron) as well as the human monoclonal anti-IL-1β neutralizing antibody canakinumab (Ilaris®; Novartis).

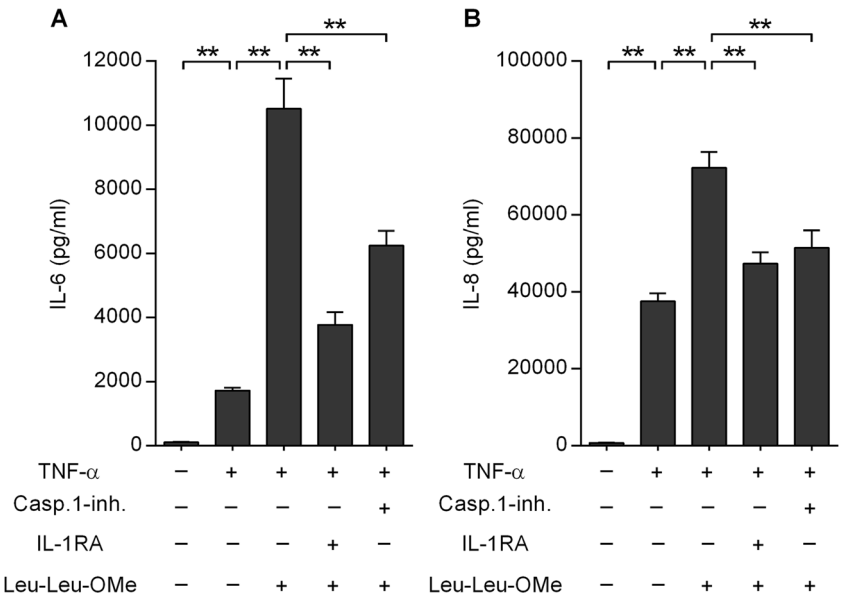

Fig. 3 The effect of Leu-Leu-OMe on the release of IL-6 and IL-8 in HUVEC cells. The extracellular concentrations of IL-6 (**a**) and IL-8 (**b**) were measured with ELISA. Results are shown as mean ± SEM of six parallel samples/group. ** denotes $P < 0.01$, Mann–Whitney *U*-test

Our present data is further support for the important role of inflammasome activation in the endothelium i.e. we demonstrated that the released IL-1β further promotes inflammation by inducing the production of IL-6 and IL-8. IL-6 is an acute phase cytokine, which is known to contribute to age-related functional decline, morbidity, and mortality (Minciullo et al. 2016). IL-8, in turn, is a chemokine attracting neutrophils to the sites of inflammation and promoting angiogenesis (Ghasemi et al. 2011).

In conclusion, our findings confirm that lysosomal homeostasis is a key factor regulating inflammasome-mediated host defence in endothelial cells. The autophagy-inflammasome axis may represent a novel therapeutic target in metabolic and age-related diseases.

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