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Nitro-oleic acid inhibits vascular endothelial inflammatory responses and the endothelial-mesenchymal transition

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ABSTRACT

Background: Inflammatory-mediated pathological processes in the endothelium arise as a consequence of the dysregulation of vascular homeostasis. Of particular importance are mediators produced by stimulated monocytes/macrophages inducing activation of endothelial cells (ECs). This is manifested by excessive soluble pro-inflammatory mediator production and cell surface adhesion molecule expression. Nitro-fatty acids are endogenous products of metabolic and inflammatory reactions that display immuno-regulatory potential and may represent a novel therapeutic strategy to treat inflammatory diseases. The purpose of our study was to characterize the effects of nitro-oleic acid (OA-NO_2) on inflammatory responses and the endothelial-mesenchymal transition (EndMT) in ECs that is a consequence of the altered healing phase of the immune response.
Methods: The effect of OA-NO_2 on inflammatory responses and EndMT was determined in murine macrophages and murine and human ECs using Western blotting, ELISA, immunostaining, and functional assays.
Results: OA-NO_2 limited the activation of macrophages and ECs by reducing pro-inflammatory cytokine production and adhesion molecule expression through its modulation of STAT, MAPK and NF-κB-regulated signaling. OA-NO_2 also decreased transforming growth factor-β-stimulated EndMT and pro-fibrotic phenotype of ECs. These effects are related to the downregulation of Smad2/3.
Conclusions: The study shows the pleiotropic effect of OA-NO_2 on regulating EC-macrophage interactions during the immune response and suggests a role for OA-NO_2 in the regulation of vascular endothelial immune and fibrotic responses arising during chronic inflammation.
General significance: These findings propose the OA-NO_2 may be useful as a novel therapeutic agent for treatment of cardiovascular disorders associated with dysregulation of the endothelial immune response.

1. Introduction

Vascular inflammation is one of the key pathological processes involved in the development of cardiovascular disorders, the major cause of mortality in developed countries. Understanding that inflammation contributes to the progression of various pathological states related to cardiovascular diseases including atherosclerosis, thrombosis, endothelial dysfunction, and fibrosis is crucial for gaining an insight into the mechanisms underlying these conditions and for identifying new pharmacological targets [1].

Innate immune cell activation is a characteristic feature of inflammatory vascular disorders. In particular, the activation of monocytes/macrophages plays a significant role in chronic inflammation inception in vessels. During the early phase of inflammation, T-cells and NK-cells predominantly secrete interferon-γ (IFN-γ), macrophages release tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and reactive

Abbreviations: α-SMA, α-Smooth muscle actin; DMEM, Dulbecco's Modified Eagle's Medium; EC, endothelial cell; EndMT, endothelial-mesenchymal transition; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; FSP1, fibroblast-specific protein 1; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP-5, murine monocyte chemoattractant protein; NF-κB, nuclear factor-κ B; NO_2-FA, nitro-fatty acid; OA-NO_2, nitro-oleic acid; PPARγ, peroxisome proliferator-activated receptor γ; RANTES, regulated on activation normal T cell expressed and secreted; STAT, signal transducer and activator of transcription; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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oxygen and nitrogen oxide-derived species, inducing pro-inflammatory phenotypes in both immune and endothelial cells (ECs). In the later phase, macrophage-derived transforming growth factor (TGF-β) is produced [2].

The activation of endothelium is a hallmark of vascular inflammatory disorders and plays a central role in mediating structural changes in the vasculature [3]. ECs represent a heterogeneous population that actively participates in both innate and adaptive immune responses, being one of the first cell types to encounter foreign pathogens and endogenous danger signals [4]. During acute and chronic inflammation, pro-inflammatory signaling pathways are triggered in ECs. Subsequently, secreted pro-inflammatory cytokines and chemokines, adhesion molecules (such as intercellular adhesion molecule-1, ICAM-1, and vascular cell adhesion molecule-1) on the surface of ECs, and the increased permeability of the vessel wall facilitate leukocyte transmigration to the site of inflamed or damaged tissue [5,6]. This effect was shown to be time-dependent [4]. While regulated on activation normal T cell expressed and secreted (RANTES) and interleukin-6 (IL-6) are potent chemoattractants for T lymphocytes and neutrophil granulocytes, murine monocyte chemoattractant protein (MCP-5, the structural and functional homologues of human MCP-1) is a chemoattractant for blood monocytes [6,7]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates pro-inflammatory M1 macrophages to induce fibrosis progression [8]. Moreover, during the activation of ECs, nitric oxide (·NO) production is also dysregulated [9].

TGF-β is a multifunctional modulator of physiologic processes such as wound healing, with its overproduction triggering pathological vascular remodeling and fibrotic changes through the induction of EndMT [10–13]. In fibrotic tissue, fibroblasts are of different origins [14]. For example, cardiac and renal fibroses are associated with the emergence of fibroblasts originating from ECs (ECs are estimated to participate in more than 25% of fibroblast formation), supporting that EndMT could be involved in fibrosis development [11,14]. Interestingly, macrophages releasing TGF-β (regulatory macrophages) promote fibroblast/myofibroblast switch as well as EndMT [15–18]. While in the early phase of fibrotic changes, cytoplasmic protein fibroblast-specific protein 1 (FSP1, also known as S100A-4) is produced, in latter phases, structural proteins including α-smooth muscle actin (α-SMA) are overexpressed [11]. Smads are one of the major downstream cytoplasmic mediators of TGF-β signaling in ECs [10–13]. The role of Smad3 in TGF-β-induced EndMT was also documented in different types of ECs, whereas the function of Smad2 in fibrosis induction in response to TGF-β is still not clear [10,19].

Fatty acid nitroalkene derivatives (NO_2-FAs) are endogenously generated by nitrating species formed by the acidic conditions of digestion and as a consequence of oxidative inflammatory reactions of nitrogen oxides. This latter mechanism can serve as an adaptive response to oxidative stress, since the physiological concentrations (~1 μM) that are generated clinically [20–22] are sufficient to mediate pleiotropic signaling responses that promote the resolution of inflammation [23]. Recent evidence suggests that these pluripotent signaling mediators can, as a consequence of post-translational protein modification, regulate many physiological and pathological processes by affecting a whole range of mammalian cell functions (*e.g.* the activation of macrophages and neutrophils, vasorelaxation, angiogenesis and platelet aggregation) [2,3,22,24].

Placing special focus on the modulation of macrophage functions [2,25] we now elucidate the effects of nitro-oleic acid (OA-NO_2) on inflammation and the EndMT responses of ECs. We hypothesize that OA-NO_2 inhibits pathological tissue remodeling induced by the activation of macrophages and ECs during chronic inflammation. Thus the effects of OA-NO_2 on macrophage-mediated EC inflammatory responses and EC-derived EndMT were studied in murine ECs stimulated by IFN-γ, TNF-α, IL-1β TGF-β, human ECs stimulated by TNF-α, and in bacterial endotoxin-activated macrophages.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). OA-NO_2 ((E)-9- and 10-nitro-octadec-9-enoic acid) was diluted to a 100 mM concentration in methanol and stored at −80 °C. Before each experiment, a 10 mM solution of OA-NO_2 in methanol was prepared from the stock solution and diluted in Dulbecco's Modified Eagle's Medium (DMEM; PAN-Biotech, Aidenbach, Germany) to obtain 100 μM of OA-NO_2, which was used immediately for cell culture experiments. All stock solutions were prepared and stored in sterile, low-binding tubes [2].

2.2. Cell culture and treatment

Murine pancreatic endothelial MS-1 cells and peritoneal RAW 264.7 macrophages (ATCC, Manassas, VA, USA) were grown in DMEM with 10% low endotoxin fetal bovine serum (FBS; PAA, Pasching, Austria) and 1% Penicillin/Streptomycin. Human umbilical vein endothelial cells (HUVEC) from Lonza [26] were cultivated in EGM Bullet Kit medium (Lonza).

ECs were treated with 1.0 μM OA-NO_2 with or without cytokines – IFN-γ (50 ng/ml), IL-1β (5 ng/ml), TNF-α (10 ng/ml), or TGF-β (10 ng/ml) – for different time periods (20 min–48 h). The inflammatory phenotype in macrophages was induced using bacterial lipopolysaccharide (LPS, 100 ng/ml) treated for 24 h. Before each experiment, ECs and macrophages were cultured in complete media as indicated above. Two hours before the start of experiments, the complete medium was replaced with DMEM with 2% of FBS or FBS-free DMEM. In long-term experiments (6 d), medium and treatments were renewed regularly after 2 days. The final concentration of OA-NO_2 used for all experiments (1.0 μM) was selected on the basis of its physiological relevance and our previous experience [2,20]. OA-NO_2 was applied alone or together with cytokines. Cell viability was measured by ATP Cell Viability test (BioThema, Handen, Sweden) [27]; no effect of cytokines or OA-NO_2 exposure was detected (data not shown).

2.3. Determination of relative cytokine and chemokine levels

Commercially available mouse cytokine ELISA kits (R&D Systems, Minneapolis, MN, USA; eBioscience, San Diego, CA, USA) were used for the determination of cytokines in cell supernatants of ECs as well as cytokines produced by macrophages. These analyses were performed according to the supplier's instructions.

2.4. Detection of protein expression

The expression of proteins was detected in cell lysates after 20 min–24 h treatments. Primary rabbit antibodies against ICAM-1, Smad2, phospho-Smad2 (Ser465/467), Smad3, phospho-Smad3 (Ser423/425), signal transducer and activator of transcription 1 (STAT1), phospho-STAT1 (Tyr701), STAT3, phospho-STAT3 (Tyr705), c-Jun. N-terminal kinase (SAPK/JNK), phospho-SAPK/JNK (Thr183/Tyr185), extracellular signal-regulated kinase (ERK1/2), phospho-ERK1/2 (Thr202/Tyr204), p38 mitogen-activated protein kinase (MAPK), phospho-p38 MAPK (Thr180/Tyr182), nuclear factor-κB (NF-κB) p65, phospho-NF-κB p65 (Ser536), endothelial nitric oxide synthase (eNOS), phospho-eNOS (Ser1777), GAPDH; primary mouse antibodies against α-SMA, inducible nitric oxide synthase (iNOS), β-actin; and appropriate secondary IgG antibodies (Santa Cruz Biotechnology, Dallas, TX, USA and Cell Signaling Technology, Danvers, MA, USA) were used. Relative levels of proteins were quantified by scanning densitometry using the ImageJ program (National Institutes of Health, Bethesda, MD, USA) with the individual band density value expressed in arbitrary units (optical density, OD).