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NecroX-7 reduces necrotic core formation in atherosclerotic plaques of *Apoe* knockout mice

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ABSTRACT

Background and aims: A large necrotic core is a key feature of atherosclerotic plaque instability. Necrotic cellular debris accumulates in the lipid-rich core and promotes inflammation, destabilization and ultimately rupture of the plaque. Although the role of necrosis in atherosclerosis is rather clear-cut, not many strategies have been performed up till now to specifically target plaque necrosis. In the present study, we tested the plaque stabilizing potential of NecroX-7, a novel compound with antioxidative and anti-necrotic properties.

Methods: Male apolipoprotein E (*Apoe*) knockout mice were treated with NecroX-7 (30 mg/kg) or vehicle, 3 times per week, via intraperitoneal injections for 16 weeks. Meanwhile, mice were fed a western-type diet to induce plaque formation.

Results: NecroX-7 reduced total plaque burden in the thoracic aorta as compared to vehicle-treated mice, without affecting total plasma cholesterol. Plaques in the aortic root of NecroX-7-treated mice showed a significant decrease in necrotic core area, 8-oxodG, iNOS and MMP13 expression, while collagen content and minimum fibrous cap thickness were increased. Moreover, NecroX-7 treatment reduced the expression of multiple inflammation markers such as *TNFα*, *IL1β*, *iNOS*, *HMGB1* and *RAGE* in a NF-κB-dependent manner. *In vitro*, NecroX-7 prevented *tert*-butyl hydroperoxide (tBHP)-induced mitochondrial ROS formation, necrosis, iNOS expression and HMGB1 release in primary macrophages.

Conclusions: NecroX-7 improves features of plaque stability in *Apoe* knockout mice by reducing necrotic core formation, oxidative stress and inflammation, and by increasing collagen deposition and fibrous cap thickness. Therefore, NecroX-7 could be a promising pleiotropic drug for the treatment of atherosclerosis.

1. Introduction

Formation and enlargement of a necrotic core plays a key role in the pathology of unstable atherosclerotic plaques [1]. In a detailed morphometric analysis of advanced human lesions, 80% of necrotic cores appeared to be larger than 1 mm^2 and comprised >10% of the plaque area [2]. However, in two thirds of the examined ruptured plaques, the necrotic core occupied >25% of the plaque [2]. Cellular debris of necrotic cells as well as the accumulation of lipids, most likely released by necrotic foam cells, contributes to necrotic core formation [3]. Necrotic cell death is morphologically distinguishable from other forms of cell death (e.g. apoptosis) by a gain in cell volume (oncosis) and swelling of organelles, followed by rupture of the plasma membrane and release of intracellular content [4]. The necrotic debris is a major source of damage-associated molecular patterns (e.g. high mobility group box 1 [HMGB1]), pro-inflammatory cytokines (e.g. IL6), proteases (e.g. matrix metalloproteinases [MMPs]) and pro-thrombotic factors (e.g. tissue

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factor) [5] and thus can jeopardize plaque stability by promoting inflammation, plaque rupture (e.g. by thinning of the fibrous cap) and subsequent thrombosis [2]. Necrosis in atherosclerotic plaques is triggered by various stimuli including high levels of reactive oxygen species (ROS), ATP depletion and elevated intracellular calcium levels [6]. Moreover, impaired phagocytic clearance of apoptotic cells promotes secondary necrosis and expansion of the necrotic core [5,7]. Only since the late 1990's, the first conclusive evidence for necrotic cell death in human plaques was published [8,9]. Histological analysis of advanced lesions showed that the necrotic core is surrounded by dying macrophages and contains predominantly macrophage debris [3,8].

Despite the importance and potential therapeutic relevance of necrosis in human atherosclerosis, plaque necrosis has not been a subject of interest for many years. Possible reasons are the lack of reliable markers to detect necrosis and the poor availability of therapeutic agents to specifically target necrosis. Essentially, necrosis has long been considered as an accidental and uncontrolled form of cell death. However, this concept changed when researchers identified receptor-interacting protein (RIP) kinases, in particular RIPK1 and RIPK3, as key regulators in mediating programmed necrosis (also called necroptosis) [10–12]. *Ripk3*-deficient low density lipoprotein receptor (*Ldlr*) knockout mice show a significant reduction in the size of advanced plaques due to inhibition of macrophage necrosis [13], while early lesions are not affected. This finding suggests that RIPK3-mediated necroptosis promotes plaque instability in advanced plaques, but does not play a role in early plaque development.

Given that necrosis is a poorly investigated yet crucial phenomenon in atherosclerosis, we aimed to inhibit necrosis by targeting one of its main inducers, namely mitochondrial ROS. Numerous studies have demonstrated the importance of mitochondrial ROS in the progression of atherosclerosis both in patients and animal models [14–18]. High levels of ROS may lead to necrosis by causing irreversible damage of vital cellular components such as DNA, proteins and lipids. Recently, several research groups reported promising results after administration of NecroX compounds, a novel class of small molecules that exhibit antioxidative properties through ROS and RNS scavenging [19], NADPH oxidase inhibition [20] and inhibition of mitochondrial ROS generation [21]. Moreover, NecroX compounds have been shown to prevent oxidative-stress induced necrotic cell death [19,20], to inhibit HMGB1-mediated inflammatory responses [22] and to protect against myocardial necrosis after ischemia-reperfusion injury [23]. Furthermore, NecroX-7 is currently being tested in ST-segment elevated myocardial infarction (STEMI) patients undergoing percutaneous coronary intervention (https://clinicaltrials.gov, identifier NCT02070471). Based on these findings, we were encouraged to evaluate the potential plaque stabilizing effects of NecroX-7 in atherosclerosis. Our data indicate that NecroX-7 increases features of plaque stability in western-type diet fed apolipoprotein E knockout (*Apoe*$^{-/-}$) mice by reducing necrotic core formation, suppressing oxidative stress, lowering plaque inflammation and increasing collagen deposition and fibrous cap thickness.

2. Materials and methods

2.1. Mice

Male *Apoe*$^{-/-}$ mice (C57BL/6, Jackson Laboratory, stock number 002052) were fed a western-type diet (4021.90, AB Diets) for 16 weeks. Simultaneously, mice were treated with NecroX-7 (LG Life Sciences, 30 mg/kg body weight) (n = 25) or vehicle (water for injection) (n = 26) via intraperitoneal injections 3 times per week. Mice were housed in a temperature-controlled room with a 12 h light/dark cycle and food and water *ad libitum*. At the end of the experiment, mice were fasted overnight and blood was collected by cardiac puncture. Total cholesterol, LDL cholesterol and triglyceride levels were measured on a Dimension Vista® System (Siemens Healthcare Diagnostics) with reagents from the same manufacturer. Plasma levels of oxLDL and malondialdehyde (MDA) were measured by an ELISA (USCNK, SEA527Mu) and a HPLC-fluorescence detection method [24], respectively. Tissues were imbedded in Neg-50 Frozen Section Medium and stored at −80 °C or fixed in formalin 4% for 24 h before paraffin imbedding. For *in vitro* experiments, wild-type mice (C57BL/6, Jackson Laboratory, stock number, 000664) and *Ripk3*$^{-/-}$ mice were used. *Ripk3*$^{-/-}$ mice, produced by targeted gene deletion [25], were a generous gift from K. Newton and V. Dixit (Genentech, San Francisco). All experiments were approved by the Ethical Committee of the University of Antwerp.

2.2. Histological analysis

The thoracic aorta was stained *en face* with Oil Red O (ORO, Sigma-Aldrich) to determine plaque burden. Atherosclerotic plaques located in the aortic root were analyzed by immunohistochemistry at 3 different sections sliced at equally spaced intervals (every 50 μm). The size of the necrotic core was measured on a hematoxylin-eosin (H&E) staining according to a standard method [26]. A 3000 μm^2 minimum threshold was implemented in order to avoid counting of regions that likely do not represent necrotic core areas. The size of the necrotic core was determined by measuring the area of the necrotic core divided by the total plaque size. A Sirius red (Sigma-Aldrich) staining was used for detection of total collagen. Collagen type I was detected on Sirius red-stained sections visualized under polarized light [27]. Apoptosis was determined by anti-cleaved caspase-3 (9661, Cell signaling Technology) and TUNEL (S7101, Millipore) staining. Plaques were further analyzed by immunohistochemistry with the following primary antibodies: mouse anti-α-SMC-actin (A2547, Sigma-Aldrich) and rabbit anti-MMP13 (ab39012, Abcam). The minimum thickness of the fibrous cap within each plaque was measured on an α-SMC-actin staining. Frozen sections of the plaques were analyzed with rabbit anti-Moma2 (MCA519, Serotec), rabbit anti-8-oxodG (orb10011, Biorbyt) and rabbit anti-iNOS (BML-SA200, Enzo Life Sciences). Isotype IgG controls were used as negative controls to assess non-specific background signals of the primary antibodies. Thereafter, tissue sections were incubated with species-appropriate HRP-conjugated secondary antibodies followed by 60 min of reactive ABC. 3,3'-diaminobenzidine or 3-amino-9-ethylcarbazole were used as a chromogen. All images were acquired with Universal Grab 6.1 software using an Olympus BX40 microscope and quantified with Image J software.

2.3. Cell culture

Bone marrow-derived macrophages (BMDM) were harvested by flushing bone marrow from the hind limbs of mice. Cells were cultured for 7 d in RPMI medium (Gibco Life Technologies) supplemented with 15% L-cell conditioned medium (LCCM) containing monocyte colony stimulating factor (M-CSF). To polarize BMDM into M1 macrophages, cells were incubated for 24 h with 100 ng/ml LPS (Sigma-Aldrich). To induce oxidative stress-mediated necrosis, BMDM were treated with 0.5 mol/l and 1 mmol/l *tert*-butyl hydroperoxide (tBHP, Sigma Aldrich) for 2 h and 4 h. Intracellular and mitochondrial ROS was measured using the fluorogenic marker 2′,7′-dichlorofluorescin diacetate (DCFDA, Molecular Probes) and MitoSOX Red (Molecular Probes), respectively. After incubation