Free Radical Biology & Medicine 50 (2011) 1382–1391

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

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

Original Contribution

Antioxidant signaling via Nrf2 counteracts lipopolysaccharide-mediated inflammatory responses in foam cell macrophages

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ARTICLE INFO

Article history:
Received 27 October 2010
Revised 23 February 2011
Accepted 24 February 2011
Available online 5 March 2011

Keywords:
Foam cell
OxLDL
Nrf2
C/EBP
Desensitized phagocytes
Free radicals

ABSTRACT

Inflammatory conditions and oxidative stress contribute to the development of atherosclerosis. Nuclear factor E2-related factor 2 (Nrf2) is a redox-sensitive transcription factor known for its antioxidant, anti-inflammatory, and, thus, cell-protective properties. Its role in effecting a deactivated state of oxidized low-density lipoprotein (oxLDL)-generated foam cell macrophages (FCMs), a prevailing cellular phenotype of atherosclerotic lesions, has not been investigated yet. In this study RAW264.7- or mouse peritoneal macrophage-derived FCMs showed reduced mRNA expression of proinflammatory cytokines such as IL-1β and IL-6 and an attenuated production of reactive oxygen species (ROS), as analyzed by hydroethidine in response to lipopolysaccharide (LPS) and compared to LPS-treated control macrophages. In peritoneal FCMs from Nrf2$^{-/-}$ mice (C57BL/6J), the LPS-induced proinflammatory response was restored. OxLDL induced heme oxygenase (HO)-1, which was Nrf2-dependent, and inhibition of HO-1 activity in FCMs using zinc protoporphyrin-IX allowed the cells to regain a proinflammatory phenotype. Mechanistically, oxLDL attenuated ROS-dependent activation of CCAAT/enhancer binding protein (C/EBP) family members in FCMs, thereby reducing cytokine expression. Thus, in FCMs the Nrf2/HO-1 axis intervenes in LPS signaling. ROS production is impaired, C/EBP transactivation is reduced, and consequently the expression of proinflammatory mediators is attenuated, thereby shaping a desensitized FCM phenotype. This macrophage phenotype may be important for the progression of atherosclerosis.

Atherosclerosis is a chronic inflammatory vascular disease and a leading cause of death in industrialized nations. At early stages of atherogenesis, monocytes infiltrate into the intima and differentiate into macrophages [1]. These phagocytes engulf modified low-density lipoprotein, e.g., oxidized LDL (oxLDL), to form lipid-rich foam cell macrophages (FCMs).

Macrophages exhibit plastic characteristics, adjusting their properties to an existing environment [2]. Thus, macrophages can be classically activated (M1) by proinflammatory stimuli such as interferon-γ or lipopolysaccharide (LPS) or can display an alternatively activated (M2) phenotype, when exposed to anti-inflammatory cytokines such as IL-4, IL-10, and IL-13. These two phenotypes are considered the extremes of a continuum of possible phenotypes. It becomes evident that in atherosclerotic plaques a heterogeneity of monocytes, macrophages, and FCMs exists [3]. Bouhlel et al. documented the occurrence of macrophages expressing not only M1 but also M2 markers in atherosclerotic plaques [4]. Distinct stages of atherosclerotic progression seem to account for phenotype characteristics. Early lesions contain M2 macrophages, whereas in late lesions M1 macrophages predominate [5]. Upon the first contact with modified LDL macrophages become classically activated. Consequently, these macrophages produce reactive oxygen species (ROS) and proinflammatory cytokines [6,7]. In vitro studies revealed that FCMs exposed over a long period to modified LDL display changes in gene expression and responses to inflammatory mediators that overall indicate macrophage deactivation [7]. Deactivation or desensitization of macrophages is characterized by a dampened inflammatory response toward stimuli such as LPS or oxLDL, both agonists of the Toll-like receptor (TLR) 4-signaling pathway [8,9]. Along the way, FCMs reduce cyclooxygenase-2 (COX-2) as well as inducible nitric oxide synthase (iNOS) expression and decrease the production of IL-

Abbreviations: ARE, antioxidant response element; bZIP, basic-leucine zipper; C/EBP, CCAAT/enhancer binding protein; CORM2, tricarbonyldichlororuthenium(II) dimer; COX-2, cyclooxygenase-2; DMNQ, 2,3-dimethoxy-1-naphthoquinone; DMSO, dimethyl sulfoxide; DPI, diphenyliodonium; EMSA, electrophoretic mobility-shift assay; FCM, foam cell macrophage; HE, hydroethidine; HO-1, heme oxygenase-1; iCORM2, inactivated CORM2; IL, interleukin; iNOS, inducible nitric oxide synthase; Keap-1, Kelch-like ECH-associated protein-1; LDL, low-density lipoprotein; LPS, lipopolysaccharide; NAC, *N*-acetylcysteine; NFCM, non-foam-cell macrophage; Nrf2, nuclear factor E2-related factor 2; oxLDL, oxidized LDL; PCR, polymerase chain reaction; qPCR, quantitative PCR; ROS, reactive oxygen species; TLR, Toll-like receptor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ZnPPIX, zinc protoporphyrin-IX.

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doi:10.1016/j.freeradbiomed.2011.02.036

12 or IL-1β upon LPS stimulation, compared to LPS-treated naive macrophages [10–13].

The anti-inflammatory transcription factor nuclear factor E2-related factor 2 (Nrf2), a member of the Cap'n-Collar basic-leucine zipper (bZIP) transcription factor family, is activated in immune cells during atherosclerosis [14]. Under resting conditions Nrf2 is retained in the cytosol, bound to Kelch-like ECH-associated protein-1 (Keap-1), and is marked for ubiquitin-dependent proteasomal degradation. Oxidative and electrophilic stress modifies Keap-1 and stabilizes Nrf2 by blocking its degradation. De novo synthesized Nrf2, not marked for degradation, translocates to the nucleus where it binds to the antioxidant response elements (AREs) of target genes [15]. Additionally, phosphorylation of Nrf2 regulates its subcellular localization and activity. Phosphorylation at serine 40 through protein kinase C enhances its activity, whereas phosphorylation of tyrosine 568 in the nucleus via glucogen synthase kinase-3β results in its nuclear export [16]. The lipid peroxidation end product 4-hydroxynonenal and phospholipids are natural components of oxLDL, known to activate Nrf2 [14,17]. After its stabilization Nrf2 translocates to the nucleus and activates transcription of antioxidative and detoxifying enzymes, such as heme oxygenase-1 (HO-1), peroxiredoxin-1, or glutamate–cysteine ligase subunits and induces the scavenger receptor CD36 [14,18].

Until now, no data on the role of Nrf2 in the deactivation of FCMs exist. In this study we provide evidence that Nrf2 activation by oxLDL attenuates proinflammatory cytokine expression upon TLR4 activation by LPS. Mechanistically, this is caused by increased HO-1 expression, reduced ROS production, and concomitant CCAAT/enhancer binding protein (C/EBP) transrepression in response to LPS. An increase in Nrf2 activity adds to the desensitized phenotype of FCMs to counteract an immoderate inflammation state of macrophages early in atherosclerosis.

Materials and methods

Materials

LPS (*Escherichia coli,* serotype 0127:B8), 2,3-dimethoxy-1-naphthoquinone (DMNQ), diphenyliodonium (DPI), *N*-acetylcysteine (NAC), tricarbonyldichlororuthenium(II) dimer (CORM2), and bilirubin were purchased from Sigma–Aldrich (Deisenhofen, Germany). Hydroethidine (HE) was ordered from Molecular Probes (Invitrogen, Karlsruhe, Germany) and zinc protoporphyrin-IX (ZnPPIX) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For each experiment CORM2 was freshly dissolved in dimethyl sulfoxide (DMSO). Inactivation of CORM2 (iCORM2) was performed as previously described [19]. In brief, after CORM2 was dissolved in DMSO, it was incubated for 18 h at 37 °C in a 5% (v/v) CO_2 humidified atmosphere.

For Western analysis, electrophoretic mobility-shift assay (EMSA), and immunofluorescence microscopy the following antibodies were used: anti (α)-C/EBPβ, α-C/EBPδ, α-lamin A/C, α-Nrf2, and α-KSRP were from Santa Cruz Biotechnology. α-HO-1 antibody was from Enzo Life Sciences (Lörrach, Germany) and α-tubulin was from Sigma–Aldrich. Secondary antibodies for Western analysis (IRDye800-labeled α-mouse, IRDye800-labeled α-rabbit) were obtained from Li-Cor Biosciences (Bad Homburg, Germany). Secondary antibodies for immunofluorescence were purchased from BD Biosciences (Heidelberg, Germany). Oligonucleotides were obtained from Biomers (Ulm, Germany) except as noted otherwise.

Cell culture and isolation of peritoneal mouse macrophages

The mouse monocyte/macrophage cell line RAW264.7 and peritoneal mouse macrophages were maintained in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% (v/v) heat-inactivated fetal calf serum at 37 °C and 5% (v/v) CO_2.

$Nrf2^{-/-}$ mice (C57BL/6J) were kindly provided by Professor M. Yamamoto (Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Japan) [20]. Genotype determinations were done by PCR of tail DNA. Peritoneal macrophages (PMs) were prepared by injecting ice-cold PBS intraperitoneally and cells from three to five mice were pooled, if necessary. After incubation for 2 h, nonadherent cells were removed and remnant cells were cultured for 5 days before performing experiments. All procedures performed on these mice followed the guidelines of the Hessian animal care and use committee.

To differentiate macrophages into FCMs, RAW264.7 cells or peritoneal mouse macrophages were incubated for 15 h with 40 μg/ml oxLDL before LPS stimulation (1 μg/ml) for the indicated times.

LDL isolation and treatment

Human LDL (day 5, 1.02–1.06 g/ml) was isolated from plasma (DRK Blutspendedienst Baden-Württemberg-Hessen) by sequential ultracentrifugation. Copper-mediated oxidation of LDL was performed as described earlier [21].

Analysis of intracellular cholesterol

Intracellular cholesterol was measured as previously described [22]. Briefly, macrophages were incubated for 15 h with 40 μg/ml oxLDL. Intracellular cholesterol was measured using a commercially available kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

EMSA

Nuclear extracts from RAW264.7 macrophages were prepared and EMSA was performed as described earlier [23]. 5′-IRDye700-labeled oligonucleotides for C/EBP DNA-binding studies were ordered from Metabion (Martinsried, Germany) and designed according to a C/EBP-like consensus sequence. Sequences were as follows: C/EBP-forward, 5′-IRDye700-ACAATTGTGCAATCTTAAT-3′; C/EBP-reverse, 5′-IRDye700-ATTAAGATTGCACAATTG-3′.

C/EBP-decoy experiment

C/EBP-decoy oligonucleotides were used as described before [24]. Phosphothiorate stabilized 5′-terminal fluorescein-labeled oligonucleotides were used. The sequences were as follows: C/EBP forward, 5′-ACAATTGTGCAATCTTAAT-3′, and C/EBP reverse, 5′-ATTAAGATTGCACAATTGT-3′. For the mutated oligonucleotides the sequences were C/EBP mut-forward, 5′-CAA**GATA**TCAATCTTAAC-3′, and C/EBP mut-reverse, 5′-GTTAAGATTGA**TATC**TTG-3′.

Determination of ROS

ROS production was determined by measuring intracellular superoxide anion formation using the HE dye. Therefore 1×10^5 RAW264.7 cells per well were seeded on coverslips in a six-well plate 1 day before performing the experiment. After preincubation (15 h) with oxLDL, the cells were stimulated for 1 h with LPS to induce ROS production. The medium was changed and cells were incubated in PBS with 5 μM HE for 30 min in the dark at 37 °C, 5% (v/v) CO_2. Afterward the cells on coverslips were washed with ice-cold PBS, followed by fixation for 1 h at room temperature with 4% (w/v) paraformaldehyde. Before the coverslips were mounted on glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA), nuclear staining was done using 4′,6-diamidino-2-phenylindol (DAPI). Fluorescence staining was