Free Radical Biology and Medicine 87 (2015) 58–68

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Molecular hydrogen stabilizes atherosclerotic plaque in low-density lipoprotein receptor-knockout mice

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ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form
30 May 2015
Accepted 4 June 2015
Available online 25 June 2015

Keywords:
Hydrogen
Plaque stability
Macrophage apoptosis
Endoplasmic reticulum stress
NF-E2-related factor-2
Free radicals

ABSTRACT

Hydrogen (H_2) attenuates the development of atherosclerosis in mouse models. We aimed to examine the effects of H_2 on atherosclerotic plaque stability. Low-density lipoprotein receptor-knockout ($LDLR^{-/-}$) mice fed an atherogenic diet were dosed daily with H_2 and/or simvastatin. In vitro studies were carried out in an oxidized-LDL (ox-LDL)-stimulated macrophage-derived foam cell model treated with or without H_2. H_2 or simvastatin significantly enhanced plaque stability by increasing levels of collagen, as well as reducing macrophage and lipid levels in plaques. The decreased numbers of dendritic cells and increased numbers of regulatory T cells in plaques further supported the stabilizing effect of H_2 or simvastatin. Moreover, H_2 treatment decreased serum ox-LDL level and apoptosis in plaques with concomitant inhibition of endoplasmic reticulum stress (ERS) and reduction of reactive oxygen species (ROS) accumulation in the aorta. In vitro, like the ERS inhibitor 4-phenylbutyric acid, H_2 inhibited ox-LDL- or tunicamycin (an ERS inducer)-induced ERS response and cell apoptosis. In addition, like the ROS scavenger *N*-acetylcysteine, H_2 inhibited ox-LDL- or Cu^{2+} (an ROS inducer)-induced reduction in cell viability and increase in cellular ROS. Also, H_2 increased Nrf2 (NF-E2-related factor-2, an important factor in antioxidant signaling) activation and Nrf2 small interfering RNA abolished the protective effect of H_2 on ox-LDL-induced cellular ROS production. The inhibitory effects of H_2 on the apoptosis of macrophage-derived foam cells, which take effect by suppressing the activation of the ERS pathway and by activating the Nrf2 antioxidant pathway, might lead to an improvement in atherosclerotic plaque stability.

1. Introduction

Atherosclerotic plaques develop as a consequence of the accumulation of circulating lipid and the subsequent migration of inflammatory cells (macrophages and T lymphocytes) and vascular smooth muscle cells (VSMCs)[2] [1]. The plaques consist of a lipid-rich core, a fibrous cap composed of collagen and extracellular matrix, and VSMCs [2,3]. Recent studies have emphasized that acute coronary syndromes are caused by plaque rupture; thus current therapies predominantly focus on stabilization of plaques rather than plaque regression [4]. Macrophage apoptosis occurs throughout all stages of atherosclerosis and plays important roles in plaque regression and plaque instability [5]. In early lesions, macrophage apoptosis is associated with diminished plaque cellularity and decreased lesion progression. In late lesions, however, a number of factors may contribute to defective phagocytic

Abbreviations: CHOP, C/EBP homologous protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DC, dendritic cell; PERK, double-stranded RNA-activated protein kinase-like ER kinase; ER, endoplasmic reticulum; eIF2α, eukaryotic translation initiation factor 2α; HO-1, heme oxygenase-1; LDH, lactate dehydrogenase; LDL-C, low-density lipoprotein cholesterol; LDLR−/−, low-density lipoprotein receptor-knockout; MMP-9, matrix metalloproteinase-9; NAC, *N*-acetylcysteine; Nrf2, NF-E2-related factor-2; ox-LDL, oxidized low-density lipoprotein; PBA, 4-phenylbutyric acid; NQO1, quinine oxidoreductase 1; ROS, reactive oxygen species; Treg, regulatory T cells; TC, total cholesterol; TM, tunicamycin; VSMC, vascular smooth muscle cell

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.06.018
0891-5849/

clearance of apoptotic macrophages, leading to a proinflammatory response, accompanied by the generation of the necrotic core, promoting further inflammation, plaque instability, and thrombosis [6]. Thus, it is believed that inhibition of macrophage apoptosis may be a useful therapeutic strategy directed against plaque instability [6,7].

Hydrogen (dihydrogen; H_2), as the lightest and most abundant chemical element, is considered a novel antioxidant that can reduce oxidative stress [8]. Consequently, hydrogen gas has come to the forefront of therapeutic medical gas research. Accumulated evidence in a variety of biomedical fields using clinical and experimental models for many diseases proves that H_2, administered through either gas inhalation or consumption of an aqueous H_2-containing solution, can act as a feasible therapeutic strategy in different oxidative stress-injured disease models. For example, supplementation with H_2-rich water was demonstrated to have a beneficial role in prevention of type 1 and type 2 diabetes and insulin resistance [9,10], chronic liver inflammation [11], acute liver injury [12], and focal brain ischemia/reperfusion injury [8]. In addition, we have reported that administration of H_2-saturated saline or water decreases plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels in high-fat diet-fed hamsters [13] and patients with potential metabolic syndrome [14]. Also, we [15] and others [16] have found that consumption of H_2-saturated saline or water prevents atherosclerosis in apolipoprotein E-knockout mice. However, the effect of H_2 on atherosclerotic plaque stability still remains elusive. Given the reported antiapoptotic, antioxidative, and anti-inflammatory effects of H_2, we hypothesized that H_2 might stabilize atherosclerotic plaque by suppressing macrophage apoptosis. In the present study, we examined the effects of H_2 on atherosclerotic plaque stability and the underlying mechanisms in low-density lipoprotein receptor-knockout ($LDLR^{-/-}$) mice and macrophage-derived foam cell models.

2. Materials and methods

2.1. H_2-saturated medium and H_2-saturated saline preparation

The H_2-saturated medium and H_2-saturated saline were prepared as previously described [17]. Briefly, H_2 was dissolved in Dulbecco's modified Eagle's medium (DMEM) or saline for 2 h under high pressure (0.4 MPa) to the supersaturated level using a self-designed hydrogen-rich-water-producing apparatus. The saturated hydrogen medium or saline was stored under atmospheric pressure at 4 °C in an aluminum bag with all the air removed. Hydrogen-rich medium and saline were prepared fresh every week to ensure a constant hydrogen concentration of more than 0.6 mM as measured by a H_2 sensor (Unisense, Denmark).

2.2. Animals and experimental design

$LDLR^{-/-}$ mice were kindly provided by Professor Jie Pan at Shandong Normal University and bred in the laboratory at TaiShan Medical University. All experiments were approved by the laboratory animal ethics committee of Taishan Medical University and followed national guidelines for the care and use of animals. At 8–9 weeks of age, the 80 male $LDLR^{-/-}$ mice fed a high-fat diet (15.8% fat and 1.25% cholesterol) were randomly divided into five groups ($n=14$ each group): the control group (vehicle treated), the low-dose (0.5 ml/kg/day) H_2-saturated saline-treated group, the high-dose (5 ml/kg/day) H_2-saturated saline-treated group, the simvastatin (5 mg/kg/day) group, and the combination group of low-dose H_2 and half-dose simvastatin (0.5 ml/kg/day H_2 and 2.5 mg/kg/day Sim). Vehicle (saline) or H_2-saturated saline was intraperitoneally injected once daily and vehicle (water) or simvastatin was administered intragastrically once daily for 28 weeks.

2.3. Plaque analysis

The proximal aorta attached to the heart was used to prepare cross sections. Cryosections (8 μm) were cut from the site where the aorta valve cups appear at the aortic root and collected on glass slides for the subsequent analyses. Atherosclerotic plaques were investigated at five independent sections, each separated by 80 μm. Oil red O staining and trichrome staining (Sigma, HT15) were performed to determine lipid-rich cores and collagen tissues, respectively. Immunofluorescence staining was done using anti-α-smooth muscle cell (SMC)–actin (Abcam, ab5694) antibody, anti-MOMA-2 (Serotec, MCA519G) antibody, and anti-MMP-9 (Santa Cruz, sc-6840) antibody to mark the areas of SMCs, macrophages, and MMP-9, respectively. Corresponding areas were analyzed and quantified using Image-Pro Plus software. Plaque stability was evaluated by comparing the percentages of the above plaque components in the entire plaque. The histological plaque stability score was calculated as plaque stability score = (SMC area + collagen area)/(macrophage area + lipid area) [18].

For immunofluorescence staining of atherosclerotic lesions, serial aortic root cryosections were blocked with 5% normal donkey serum and incubated with the primary antibodies, including Bip (Santa Cruz), C/EBP homologous protein (CHOP; Santa Cruz), CD83 (Abcam), and FoxP3 (Abcam), overnight at 4 °C, and then the sections were incubated with Alexa Fluor 594-labeled donkey anti-rabbit or Alexa Fluor 488-labeled donkey anti-rat antibodies (Molecular Probes) for 1 h. Slides were mounted with Prolong Gold antifade reagent (Invitrogen, Carlsbad, CA, USA) and viewed using a fluorescence microscope (Olympus, Tokyo, Japan).

2.4. In situ detection of apoptotic cells

Apoptotic cells and apoptotic macrophages in plaque cryosections were determined by use of an in situ apoptosis detection kit (Roche, Indianapolis, IN, USA).

2.5. Cell culture

RAW264.7 macrophages purchased from ATCC were cultured in DMEM (Hyclone) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 2 mM L-glutamine, and antibiotics. For H_2 treatment, cells were cultured in DMEM prepared with H_2 and supplemented with 1% (v/v) FBS and 2 mM L-glutamine. The H_2 medium was changed every 6 h to maintain the H_2 concentration in the medium. For all experiments, cells cultured for 4–10 passages were used.

2.6. Isolation and oxidation of LDL

Human LDL was isolated and oxidized as described recently [19]. In brief, LDL (density 1.019–1.063 g/ml) was isolated from plasma of normolipidemic donors by sequential ultracentrifugation and incubated with 10 mmol/L $CuSO_4$ for 18 h at 37 °C. After incubation, 0.1 mmol/L ethylenediaminetetraacetic acid was added to prevent further oxidation, and the oxidized LDL was concentrated to 1 mg/ml. The extent of LDL oxidation was assessed based on its increased mobility in an agarose gel (compared with that of native LDL) and also by the presence of increased concentrations of thiobarbituric acid-reactive substances (TBARS) in the sample. Typically, ox-LDL preparations had TBARS of >30 μmol/g protein and a relative mobility index on agarose gels of 2.0–2.5 compared with native LDL. Lipoproteins were stored at 4 °C in the dark and prepared fresh every 2 weeks.