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Hydrogen peroxide inhibits proliferation and endothelial differentiation of bone marrow stem cells partially via reactive oxygen species generation

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ABSTRACT

Aims: The present study was to investigate the effect of hydrogen peroxide (H_2O_2) on bone marrow stem cells and their endothelial differentiation and the underlying mechanisms in vitro.
Main methods: Rat bone marrow multipotent adult progenitor cells (MAPCs) were used as the source of bone marrow stem cells, and treated with H_2O_2 (with the final concentration from 0 to 50 μM) with or without N-acetylcysteine (NAC, 0.1 mM). Reactive oxygen species (ROS) was measured by electron paramagnetic resonance (EPR) and fluorescent microscope. Flow cytometry and immunoblotting were used to determine apoptosis and differentiation of MAPCs.
Key findings: H_2O_2 generated a significant amount of intracellular and extracellular ROS in the culture system, substantially inhibited the proliferation of MAPCs and Oct-4 expression, and induced their apoptosis in a dose-dependent manner. Exposure to H_2O_2 also significantly attenuated the endothelial differentiation of MAPCs with reduced expression of endothelial markers CD31 and FLK-1 as well as impaired in vitro vascular structure formation. Both intracellular and extracellular ROS production from H_2O_2 were blocked by NAC. NAC treatment effectively prevented H_2O_2-induced reduction of Oct-4 expression in the cells. However, NAC treatment only partially prevented H_2O_2-induced apoptosis, and inhibition of cell proliferation and endothelial differentiation of MAPCs.
Significance: H_2O_2 exposure suppressed Oct-4 expression in MAPCs through ROS-dependent mechanism, while increasing the apoptosis of MAPCs and inhibiting their proliferation and endothelial differentiation with a mechanism partially due to ROS generation in vitro.

Introduction

Bone marrow-derived circulating endothelial progenitor cells (EPCs) play a critical role in maintaining the structural and functional integrity of the vasculature (Donahue et al., 2013; Urbich and Dimmeler, 2004). The number and function of EPCs are significantly decreased in patients with a variety of diseases including coronary artery disease, diabetes mellitus (DM) and hyperlipidemia (Donahue et al., 2013; Lin et al., 2013; Tousoulis et al., 2008; Werner et al., 2005). However, the exact mechanism(s) for impaired number and function of EPCs in DM and hyperlipidemia remains to be defined. Although DM and hyperlipidemia are two very different diseases, one important shared feature for both conditions is increased level of oxidative stress and close relation to the development of atherosclerosis (Haidara et al., 2006; Singh and Jialal, 2006).

Oxidative stress is considered as an important mechanism for the development of cardiovascular diseases including atherosclerosis (Haidara et al., 2006; Singh and Jialal, 2006). While the underlying mechanisms for the development of atherosclerosis are complex and are not fully understood, one critical part is believed to be related to vascular injury and endothelial cell dysfunction in the very early stage of the atherosclerotic process (Dimmeler and Zeiher, 2004; Widlansky et al., 2003). EPCs make significant contribution to the re-endothelialization of injured blood vessels and the formation of new vasculature (angiogenesis), as well as prevention of neointima

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formation after vascular injury (Rauscher et al., 2003; Strehlow et al., 2003; Urbich and Dimmeler, 2004; Werner et al., 2003).

Reactive oxygen species (ROS) has long been considered as a major contributor to inflammatory response, and plays an import role in oxidative stress and cell function including cell-fate signaling, proliferation, gene transcription and expression, as well as cell injury and death (Mittal et al., 2014; Pervaiz et al., 2009). Hydrogen peroxide (H_2O_2), a more stable ROS, is present in all aerobic organisms. It is formed as a product of the free radical $O_2^{\bullet-}$ by superoxide dismutase, and its production is dramatically increased during oxidative burst in inflammatory conditions including the response to the challenge of lipopolysaccharides (LPS, an endotoxin) both in vivo and in vitro (Marinho et al., 2014; Vitetta and Linnane, 2014; Wang et al., 2014). H_2O_2 plays a wide range of important roles in a variety of cells in a concentration-dependent manner. At low concentrations, H_2O_2 could activate various enzymes like phosphatases and cell signaling. However, at high concentrations, it could lead to irreversible cell damage similar to a strong oxidative stress (Breton-Romero and Lamas, 2014; Lisanti et al., 2011; Marinho et al., 2014).

The mechanism(s) for the decreased number and function of EPCs in DM and hyperlipidemia is very complex and likely multifactorial. Current efforts have been largely focused on the level of EPC survival and function. Although there are multiple sources for EPCs including the bone marrow and places outside of the bone marrow like the liver and spleen, the bone marrow is considered an important source for EPCs (Aicher et al., 2007; Sirker et al., 2009). Therefore, the number and function of EPCs could be intimately associated with the differentiation potential of bone marrow stem cells (BMSCs) into EPCs. The present study was designed to test the hypothesis that oxidative stress could inhibit endothelial differentiation of BMSCs, thus decrease the number of EPCs. We observed that H_2O_2 inhibited the expression of stem cell specific marker Oct-4 in BMSCs, their proliferation and endothelial differentiation potential in vitro. The effect of H_2O_2 on BMSCs was partially, but significantly, reversed in the presence of ROS scavenger N-acetylcysteine (NAC). The data were consistent with the hypothesis.

Materials and methods

Materials

Low glucose Dulbecco's minimal essential medium (DMEM) was from Gibco BRL (Grand Island, NY, USA). Fibronectin (FN), MCDB-201, insulin transferring selenium (ITS), linoleic acid bovine serum albumin (LA-BSA), dexamethasone, L-ascorbic acid 2-phosphate sesquimagnesium salt (L-ascorbic acid) and lipopolysaccharides (LPS) were from Sigma (St. Louis, MO, USA). Platelet-derived growth factor-BB (PDGF-BB), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) were from R&D Systems (Minneapolis, MN, USA). Leukemia inhibitory factor (LIF) was from ESGRO Chemicon (Billerican, MA, USA). Fetal bovine serum (FBS) was from Hyclone (Waltham, MA, USA). Penicillin–streptomycin solution (pen/strep) was from Cellgro (Manassas, VA, USA). Primary antibodies for Flk-1 and β-actin (rabbit) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibody for PECAM-1(CD31) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). BrdU proliferation assay kit was from Calbiochem (Calbiochem, San Diego, CA, USA). Annexin V apoptosis detection kit was from Trevigen (Gaithersburg, MD USA). CellROX oxidative stress reagents (deep red) were from Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA, US). Reactive oxygen species detection reagent (FITC) was from Invitrogen (Thermo Fisher Scientific Inc., Waltham, MA, US). CD34 AF700 and Flk-1 APC-Cy7 were purchased from Becton Dickinson Biosciences (NJ, USA).

Cell culture and endothelial differentiation

Rat bone marrow multipotent adult progenitor cells (MAPCs) were prepared and characterized in Dr. Verfaillie's lab in the Stem Cell Institute at the University of Leuven, Leuven, Belgium. Phenotypically, these cell were positive for Oct-4, Rex-1, c-Kit, and Pdgfr-a, and negative for Sca-1, CD34, CD45, Sox-2, and Nanog (Ulloa-Montoya et al., 2007). The cells were cultured at a density of 100–200 cells/cm^2 in expansion medium at 37 °C with 5% O_2, 5% CO_2, and 90% N_2 as described (Breyer et al., 2006; Ulloa-Montoya et al., 2007). To evaluate the effect of H_2O_2 on MAPCs, the cells were cultured at a density of 400 cells/cm^2 in the presence of H_2O_2 (Sigma) (from 0 to 100 μM, final concentration) for 24, 36, and 48 h (protected from light) to determine the cell growth, apoptosis, and Oct-4 expression. To determine the involvement of ROS in the actions of H_2O_2, experiments were repeated when NAC (0.1 mM, final concentration) was present.

To determine the effect of H_2O_2 on endothelial differentiation of MAPCs, the cells were induced to differentiate into endothelial cells with or without H_2O_2 as described (Breyer et al., 2006; Chu et al., 2008; Ulloa-Montoya et al., 2007). To investigate the role of ROS in mediating the effect of H_2O_2 on endothelial differentiation of MAPCs, experiments were repeated in the presence of ROS scavenger NAC (NAC) (0.1 mM, final concentration) (NAC was added into the media 5 min prior to exposure to H_2O_2). After 9 days of differentiation, the cells were collected for Western blot analysis and flow cytometry analysis to determine the expression of endothelial markers Flk-1 and CD31.

Cell proliferation assay

MAPCs were seeded in a 96-well plate at a density of 1000 cells/well with or without H_2O_2 (from 0 to 50 μM) for 12, 24, 36, and 48 h. To evaluate the effect of NAC (0.1 mM, the optimal concentration based on our preliminary experiments without impact on cell growth) on cell proliferation, NAC was mixed in the culture medium 5 min before exposure to H_2O_2. At each time point, the cells were prepared for proliferation assay using the BrdU proliferation assay kit (Calbiochem, San Diego, CA, USA) as per manufacturer's instruction. All samples were prepared in duplicates.

Cell apoptosis assay

MAPCs were planted in 10-cm dishes with 500 cells/cm^2 for apoptosis assay as per manufacturer's protocol. After 6 h of incubation, the cells were exposed to H_2O_2 (from 0 to 50 μM, final concentration) for an additional 36 h with or without of NAC (0.1 mM, final concentration). The cells were then prepared for apoptotic cell death measurement with TACS annexin V kits using flow cytometry according to the manufacturer's protocol.

Measurement of ROS formation

Production of extracellular ROS from H_2O_2 in the culture system was quantitatively determined using electron paramagnetic resonance (EPR) spectroscopy as described (Zhu et al., 2007), and intracellular ROS was detected using the ROS detection reagent 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) (Life Technologies D399) dissolved in ethanol (0.5 mg/100 μl) according to the manufacturer's instruction. H2DCFDA is a cell-permeable non-fluorescent reagent. When exposed to intracellular esterases and oxidation, it is converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). For EPR measurement, the culture medium with different concentrations of H_2O_2 (from 0 to 50 μM) was mixed with detection solution. Medium with PBS and NAC was used as background. To evaluate the effect of NAC on ROS production from H_2O_2, NAC (0.1 mM) was mixed with the culture medium 5 min prior to exposure to H_2O_2. For intracellular ROS detection, the cells were seeded in 35 mm glass bottom