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Macrophage NADPH oxidase activation, impaired cholesterol fluxes, and increased cholesterol biosynthesis in diabetic mice: A stimulatory role for D-glucose

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Abstract

Diabetes is clearly associated with accelerated atherosclerosis development, but molecular mechanisms involved in diabetes-induced atherosclerosis remain to be clarified. The aim of this study was to identify cellular mechanisms involved in diabetes-induced macrophage foam cell formation, the hallmark of early atherogenesis. Mouse peritoneal macrophages (MPMs) isolated from Balb-C streptozotocin-induced diabetic mice, exhibited significantly higher total peroxides, lipid peroxides and paraoxonase 2 (PON2) activity by 290%, 61% and 55%, respectively, compared to non-diabetic mice. In vitro studies revealed that glucose-induced oxidative stress was obtained by D-glucose, but not by L-glucose and it involved activation of the NADPH oxidase complex, and up-regulation of the macrophage PON2.

Next, MPMs isolated from Balb-C diabetic mice, compared to control Balb-C mice, demonstrated increased cholesterol content by 4.2-fold associated with increased cholesterol biosynthesis and increased uptake of oxidized LDL (Ox-LDL) by 5.9-fold and 31%, respectively. These effects on cellular cholesterol metabolism were associated with up-regulation of the scavenger receptors for Ox-LDL (CD-36 and SR-A), and of HMG-CoA reductase (cholesterol biosynthesis rate limiting enzyme). Finally, using pravastatin (inhibitor of HMG-CoA reductase) and the antioxidant Vitamin E, we have shown that D-glucose-induced macrophage oxidative stress is secondary to its stimulatory effect on macrophage cholesterol biosynthesis.

In conclusion, macrophages from diabetic mice demonstrate increased oxidative stress associated with activation of NADPH oxidase and up-regulation of cellular PON2, as well as increased macrophages cholesterol uptake and biosynthesis (increased expression of CD-36 and HMG-CoA reductase). The above mechanisms in diabetic mice could be the result of the effect of high D-glucose on macrophages.

Keywords: Diabetes; Macrophages; Atherosclerosis; Oxidative stress; Cholesterol biosynthesis

1. Introduction

Diabetes mellitus (DM) is associated with premature and accelerated atherosclerosis, and patients with DM are at 2–4 times increased risk for coronary artery diseases, which accounts for the major cause of death in these patients [1,2]. The precise mechanisms underlying the acceleration of atherosclerosis in DM is poorly understood, but it was suggested that hyperglycemia accelerates atherosclerosis by induction of vascular dysfunction, increased inflammatory burden, increased lipid peroxidation of lipoproteins, and arterial cells which lead to enhanced macrophage foam cell formation, the hallmark of early atherosclerosis [3,4].

Under oxidative stress, macrophages can generate reactive oxygen and nitrogen species (ROS and RNS, respectively) and through activation of the NADPH oxidase complex increase the production and release of superoxide ions, thus leading to extensive LDL oxidation, followed by increased uptake of the formed oxidized LDL by macrophages via their scavengers receptors (SR-A and CD-36) [5,6].

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Serum paraoxonase 1 (PON1), an HDL-associated lactonase, was shown to protect against lipid peroxidation in lipoproteins, macrophages and atherosclerotic lesions [7]. PON2, unlike PON1, is not present in the circulation, but rather distributed in cells (including macrophages) and it possesses antioxidant and anti-atherosclerotic properties [8,9]. The balance between cellular pro-oxidants (such as the NADPH oxidase complex) and antioxidants (such as PON2) in arterial wall macrophages, determines the extent of LDL oxidation. Macrophage PON2 was shown to be increased under oxidative stress, probably as a compensation mechanism [10]. Cholesterol accumulation in macrophages can result from three main processes: i.e. increased lipoproteins uptake, increased cholesterol biosynthesis and decreased cholesterol efflux from the cells. The high blood glucose levels observed in diabetes could be responsible for the deleterious effects on macrophage oxidative stress and cellular cholesterol accumulation.

Indeed, culturing of endothelial cells or smooth muscle cell in glucose enriched-media increased their ability to oxidize LDL [11]. Moreover, using macrophages and vascular tissue sections from diabetic patients, it was shown that glucose increases expression of the scavenger receptor CD36 at the translation level [12]. Recently, we have shown that in diabetic mice macrophages, as well as in macrophages that were exposed to high glucose levels, increased cellular oxidative stress, LDL oxidation and Ox-LDL uptake by macrophages, leading to foam cell formation were noted [13].

The aim of the present study was to identify mechanisms involved in diabetes-induced macrophage cholesterol accumulation and foam cell formation. For this purpose, we first analyzed macrophage oxidative stress and cellular cholesterol metabolism in diabetic mice, followed by analysis of the direct effects of glucose on macrophage oxidative stress and cellular cholesterol accumulation.

2. Methods

2.1. Mice

Balb-C mice (6 weeks old) were randomly divided into two groups:

- *Diabetic mice*: The mice were injected intraperitoneally with STZ (200 mg/kg) within 5 min of preparation. Serum glucose levels were determined within 2 weeks and mice with serum glucose levels in the range of 250–400 mg/dl were included in study group. Mice were sacrificed at 16 weeks old (after 2 months of diabetes).
- *Control mice*: Non-diabetic Balb-C mice that were sacrificed at the age of 16 weeks old.

Five Balb-C mice and five $p47^{phox-/-}$ mice (a generous gift from Dr. Stephen M. Holland, Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA) were sacrificed at 16 weeks old.

2.2. Cells

Mouse peritoneal macrophages (MPM) were harvested from the peritoneal fluid of Balb-C mice 4 days after intraperitoneal injection of thioglycolate. The cell suspension is dispensed into petri dishes and incubated in an incubator (5% CO_2, 95% air) for 2 h. The dishes are washed once with DMEM to remove non-adherent cells.

2.2.1. J-774 A.1 murine macrophage-like cell line

This cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). J-774 A.1 cells were plated at 2.5×10^5 cells/16 mm dish in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U penicillin/ml, 100 μg streptomycin/ml, and 2 mM glutamine. The cells were fed every 3 days and used for experiments within 7 days of plating [14]. For in-vitro experiments, J774 A.1 macrophages were incubated with D-glucose-enriched media (5 or 30 mM) or with 5 mM D-glucose mixed with 25 mM L-glucose, for 18 h days prior to the experiments.

2.3. Macrophage oxidative stress

Determination of cellular peroxidation by DCFH-DA flow cytometric assay was determined by incubation of the cells with DCFH-DA. Under oxidative stress, DCFH is oxidized to 2′,7′-dichlorofluorescein (DCF), which is fluorescent. Cellular fluorescence was determined with a flow cytometer (FACS-SCAN, Becton Dickinson, San Jose, CA, USA), at 510–540 nm after excitation of cells at 488 nm with an argon ion laser [15].

2.3.1. Determination of cellular lipid peroxidation

Macrophages lipid peroxides (PD) were assayed in MPM sonicate [16]. Peritoneal macrophages were suspended in PBS (6×10^6/ml) and sonicated at 80 W for 3×20 s. Cellular PD was determined in the sonicate by following incubation of the cell sonicate with lipid peroxides determination reagent using a spectrophotometer at 365 nm (Ultrospec 3000, Pharmacia Biotech, Cambridge, England).

2.3.2. Cell-mediated oxidation of LDL

Following its incubation with the cells, LDL was analyzed for its oxidation levels using the TBARS assay [17].

2.3.3. Macrophage paraoxonase 2 (PON2)

2.3.3.1. Macrophage PON2 activity. This activity was determined by analyses of cellular lactonase activity using dihydrocoumarin as a substrate [8].