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Increased macrophage cholesterol biosynthesis and decreased cellular paraoxonase 2 (PON2) expression in Δ6-desaturase knockout (6-DS KO) mice: Beneficial effects of arachidonic acid

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ABSTRACT

Objective: To analyze the possible role of arachidonic acid (AA) in macrophage cholesterol biosynthesis and in PON2 expression.
Methods and results: We used peritoneal macrophages (MPM) from the 6-DS KO mice that were fed a diet without or with AA. Macrophage cholesterol biosynthesis rate and HMGCoA-reductase mRNA levels were substantially increased, by 98% and 67%, respectively, in MPM from 6-DS KO vs. control (C57BL/6) mice. Furthermore, in the 6-DS KO vs. control mice MPM PON2 expression (mRNA and lactonase activity) was substantially decreased. In line with the above results, AA supplementation to 6-DS KO mice significantly decreased MPM cholesterol biosynthesis rate and HMGCoA-reductase mRNA levels, by 45% and by 4-fold respectively, and increased MPM PON2 lactonase activity and PON2 mRNA, by 119% and 2.3-fold, respectively.

Similarly, incubation of control mice MPM or J774A.1 with AA, significantly and dose-dependently decreased cellular cholesterol biosynthesis rate, and increased PON2 expression. These effects were specific for AA since incubation of the cells with docosahexaenoic acid (DHA, another product of 6-DS) had no significant effects on cholesterol biosynthesis rate, and on PON2 activity.
Conclusions: AA decreased macrophage cholesterol biosynthesis rate, and increased PON2 expression. These effects could protect the cells from cholesterol accumulation and oxidation, and from foam cell formation, the hallmark of early atherogenesis.

1. Introduction

The rate-limiting step in AA synthesis is the desaturation of dietary linoleic acid (LA) by 6-DS [1]. 6-DS highest level is present in human liver, but it is also expressed in other human tissues, including arterial wall [2]. Decreased 6-DS activity was suggested to be involved in the initiation and progression of atherosclerosis [3]. In addition, coronary arteries from cases of sudden cardiac death were shown to contain increased levels of LA, and decreased content of AA in their phospholipids [4].

AA is mostly esterified to membranal phospholipids, and AA metabolism involves the lipoxygenase and cyclooxygenase pathways for the formation of leukotrienes, thromboxanes and prostaglandins [5]. AA interacts directly with phospholipids, phospholipases, G-proteins, protein kinases and ion channels. Furthermore, AA and its various metabolites can act at the level of the nucleus, to affect the transcription of a variety of genes including those involved in lipid metabolism [6–9]. AA and its prostaglandins thus may affect atherosclerosis development.

Macrophage cholesterol accumulation and foam cell formation are the hallmark of early atherogenesis [10]. Several macrophage properties are associated with atherogenesis, i.e. cholesterol biosynthesis [11,12], and cellular expression of the antioxidant enzyme – PON2 [13]. In order to study the effect of AA on these macrophage properties, there is a need to dissect between the essential fatty acids LA and AA. The establishment of the 6-DS KO mice model enabled this requirement, as the lack of 6-DS allows us to study the effect of lacking AA, without the need to eliminate also LA [14].

PON2 was shown to possess anti-atherogenic characteristics [15,16], and indeed in PON2-deficient mice there is increased atherosclerosis development [17]. We have previously shown that macrophage cholesterol level affects PON2 expression [18–20]. In macrophages from atherosclerotic hypercholesterolemic patients, there is increased cholesterol mass and decreased PON2 expression and statin therapy reverse this phenomenon [18]. Similarly, incubation of macrophages with acetylated or aggregated LDL leads to cellular cholesterol accumulation (both esterified and unesterified)

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and inhibition of PON2 expression [18,19]. In contrast, in unesterified cholesterol-enriched macrophages, PON2 expression is upregulated [20].

Thus, the present study focused on the possible role of AA in macrophage cholesterol biosynthesis and in PON2 expression. For this purpose we used several systems: peritoneal macrophages from the 6-DS KO mice that received a diet without or with AA supplementation, as well as, cell cultured macrophages that were incubated with either 6-DS specific inhibitor (SC-26196), or directly with AA.

2. Methods

The methods are described in detail under the "Supplemented file."

2.1. Generation of Δ6-desaturase (6-DS) KO mice

Generation of Δ6-desaturase-null mice was described previously [14]. Both the control (C57BL/6) and the 6-DS KO mice received for 3 months the AIN-93G diet. This diet includes 3.6% linoleic acid (percent of total weight), but no arachidonic acid [21]. Another group of mice received the AIN-93G diet supplemented with 0.2% arachidonic acid (percent of total weight). All animal work was approved by the University of Illinois Institutional Animal Care and Use Committee, and by the Committee for Supervision of Animal Experiments and complied with the Guide for Care and Use of Laboratory animals, the Technion – Israel Institute of Technology, Haifa.

2.2. Serum paraoxonase 1 (PON1) activities

Arylesterase activity (phenyl acetate hydrolysis), paraoxonase activity (paraoxon hydrolysis) and lactonase activity (dihydrocoumarin hydrolysis) were measured as previously described [22].

2.3. Cells

2.3.1. Mouse peritoneal macrophages (MPM)

MPM were harvested after intraperitoneal injection of thioglycolate (40 g/L).

2.3.2. J774A.1 macrophages

J774A.1 murine macrophage cells were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD).

Both MPM and J774A.1 were grown in DMEM + 0.2% BSA. AA, DHA or LA were dissolved in 100% ethanol, and were incubated with the cells 20 h at 37 °C in DMEM + 0.2% BSA. Control cells were incubated with similar volume of ethanol, which did not exceed 0.1%. The cellular protein was determined by the Lowry assay [23].

2.3.3. Macrophage arachidonic acid (AA) levels

Cellular AA levels were determined in MPM harvested from control or from 6-DS KO mice, and in control mice MPM that were incubated with LA ± SC-26196, by HPLC. Cellular AA concentration was calculated using a calibration curve of pure AA, and is expressed as ng AA/mg cell protein.

2.3.4. Macrophage peroxides content (DCFH assay)

Cellular total peroxide levels were determined by the flow-cytometric assay with dichlorofluorescin-diacetate (DCFH-DA, [24]).

2.3.5. Macrophage cholesterol content

The amount of cellular total cholesterol, unesterified cholesterol or esterifies cholesterol was determined in the cells' lipid extract using a commercial kit.

2.3.6. Macrophage cholesterol biosynthesis

Cellular cholesterol biosynthesis was assayed by thin layer chromatography (TLC) after incubation of the cells with [^{3}H]-acetate (1 mCi/L).

2.3.7. Macrophage PON2 lactonase activity

Macrophage PON2 lactonase activity was determined spectrophotometrically at 412 nm as the hydrolysis of 5-thiobuthyl-butyrolactone (TBBL, [25]).

2.3.8. PON2 mRNA expression determination by reverse transcriptase quantitative polymerase chain reaction (Q-PCR)

Total RNA was extracted and the amount of GAPDH and PON2 mRNA was measured by quantitative PCR. The primers and probes for human PON2, and GAPDH were designed by Primer Design (South Hampton, UK).

PON2 sense primer CGACTTAAAGCCTCCAGAGAA
PON2 antisense primer GGGAATTTTAGACCCACACTAAA
PON2 double dye TaqMan probe TAGACCTTCCACACTGCCACCTGA

2.3.9. 6-DS and HMGCoA-reductase mRNA expression by RT-PCR

Total RNA was extracted, and RT products were subjected to PCR amplifications using specific primers.

HMGCoA-reductase sense primer GACACTTACAATCTGTATGATG
HMGCoA-reductase antisense primer CTTGGAGAGGTAAAACT-GCCA
6-DS sense primer TCAAAACCAACCACCTGTTCTTC
6-DS antisense primer GATGAACCAGGCAAGGCTTTC

2.4. Statistics

Each separate experiment was performed in triplicate, and each individual experiment was replicated three times ($n = 3$) in order to achieve statistical meaning. Statistical analyses used Student's *t*-test for comparing differences between the two groups, and a one-way ANOVA, which was followed by the Student–Newman–Keuls test for comparing differences between multiple groups. Results are given as mean ± SD.

3. Experimental results

3.1. Δ6-Desaturase (6-DS) expression in macrophages

MPM express 6-DS mRNA, almost like adrenal and liver tissues. J774A.1 macrophage cell line also expresses 6-DS, though to a much less extent (supplemented Fig. 1A). Furthermore, incubation of MPM with LA significantly increased the cellular AA content, compared to MPM incubated with no additions, indicating high 6-DS enzymatic activity in these cells (supplemented Fig. 1B). Upon incubation of control mice MPM with LA in the presence of 6-DS specific inhibitor SC-26196 [26], the conversion of LA to AA was completely abolished (supplemented Fig. 1B).

3.2. Serum cholesterol concentration and serum PON1 activities in 6-DS KO mice

We used 6-DS KO and control (C57BL/6) mice that were fed with AIN diet (with no AA), or with AIN diet + AA, and analyzed their serum cholesterol concentration and serum PON1 activities. Serum total cholesterol and HDL-cholesterol levels were similar in both the control and the 6-DS KO mice (Table 1). Serum PON1 arylesterase, paraoxonase and lactonase activities were all decreased by 33%, 37% and 42%, respectively, in 6-DS KO vs. control