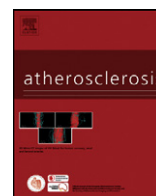




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Increased macrophage cholesterol biosynthesis and decreased cellular paraoxonase 2 (PON2) expression in Δ 6-desaturase knockout (6-DS KO) mice: Beneficial effects of arachidonic acid

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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.

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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 $\Delta 6$ -desaturase (6-DS) KO mice

Generation of $\Delta 6$ -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 \pm 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 dichlorofluorescein-diacetate (DCFH-DA, [24]).

2.3.5. Macrophage cholesterol content

The amount of cellular total cholesterol, unesterified cholesterol or esterified 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 [³H]-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-thiobutylbutyrolactone (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 GGAATTTTAGACCCACTAAA
PON2 double dye TaqMan probe TAGACCTCCACTGCCACTGA

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 CTTGGAGAGGTAAAAC-TGCCA

6-DS sense primer TCAAAACCAACCCTGTTCTTC
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 \pm SD.

3. Experimental results

3.1. $\Delta 6$ -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

Table 1
Serum paraoxonase 1 (PON1) activity and serum cholesterol concentration in $\Delta 6$ -desaturase (6-DS) KO mice supplemented with AIN diet or with AIN diet + AA.

Mice group	Control (C57BL/6) mice AIN diet	6-DS KO mice AIN diet	6-DS KO mice AIN diet + AA
Total cholesterol (mg/dl)	93 ± 7	89 ± 9	102 ± 4
HDL-cholesterol (mg/dl)	61 ± 3	68 ± 5	61 ± 7
PON1 arylesterase activity (units/ml)	257 ± 20	172 ± 12*	254 ± 7*
PON1 paraoxonase activity (units/ml)	548 ± 55	345 ± 37*	438 ± 10*
PON1 lactonase activity (units/ml)	10.5 ± 0.4	6.0 ± 0.5*	8.5 ± 1.0*

* $p < 0.01$, 6-DS KO vs. control mice.

$p < 0.01$, 6-DS KO mice on AIN diet + AA vs. 6-DS KO mice on AIN diet.

mice (Table 1). Supplementation of 6-DS mice with the control AIN diet + AA significantly increased serum PON1 arylesterase, paraoxonase and lactonase activities by 48%, 27% and 41%, respectively (Table 1).

3.3. The effect of AA on macrophage biological activities

We first compared the 6-DS KO vs. control mice macrophages, followed by analyses of the in vitro effects of AA on macrophage cholesterol biosynthesis and on cellular PON2 expression, and finally – the in vivo effects of AA supplementation.

3.3.1. Macrophage cholesterol biosynthesis in 6-DS KO mice MPM and in cell cultured macrophages

In the 6-DS KO mice MPM AA could not be detected, while in the control mice MPM, the level of AA was 2391 ± 100 ng/mg cell protein. Macrophage cholesterol biosynthesis rate (Fig. 1A), and HMGCoA-reductase mRNA levels (Fig. 1B) were substantially increased, by 98% and 67%, in MPM from 6-DS KO vs. control mice. In

accordance with these results, the total cholesterol content in MPM from 6-DS KO vs. control mice was significantly increased, by 49% (17.5 ± 1.0 vs. 12.6 ± 2.1 $\mu\text{g}/\text{mg}$ cell protein, respectively). The levels of esterified cholesterol were increased by 2.3-fold in MPM from 6-DS KO vs. control mice (9.9 ± 1.4 vs. 4.5 ± 0.5 $\mu\text{g}/\text{mg}$ cell protein, respectively), and the ratio of unesterified cholesterol/esterified cholesterol was as low as 0.28 in 6-DS KO mice MPM, and as high as 1.8 in MPM from control mice.

Incubation of control mice MPM (Fig. 1C), or J774A.1 macrophages (Fig. 1D) with LA ($2 \mu\text{M}$) significantly decreased the cholesterol biosynthesis rate by 35% or by 34%, respectively, as compared to control MPM or J774A.1 macrophages that were incubated with no addition, while incubation with SC-26196 did not affect the cholesterol biosynthesis rate in both types of macrophages (Fig. 1C and D). In contrast, and similarly to the in vivo results, incubation of MPM or J774A.1 with LA together with the 6-DS specific inhibitor SC-26196, resulted in a significant increment in cellular cholesterol biosynthesis rate, by 30% or 37%, respectively, as compared to MPM or J774A.1 incubated only with LA (Fig. 1C and D).

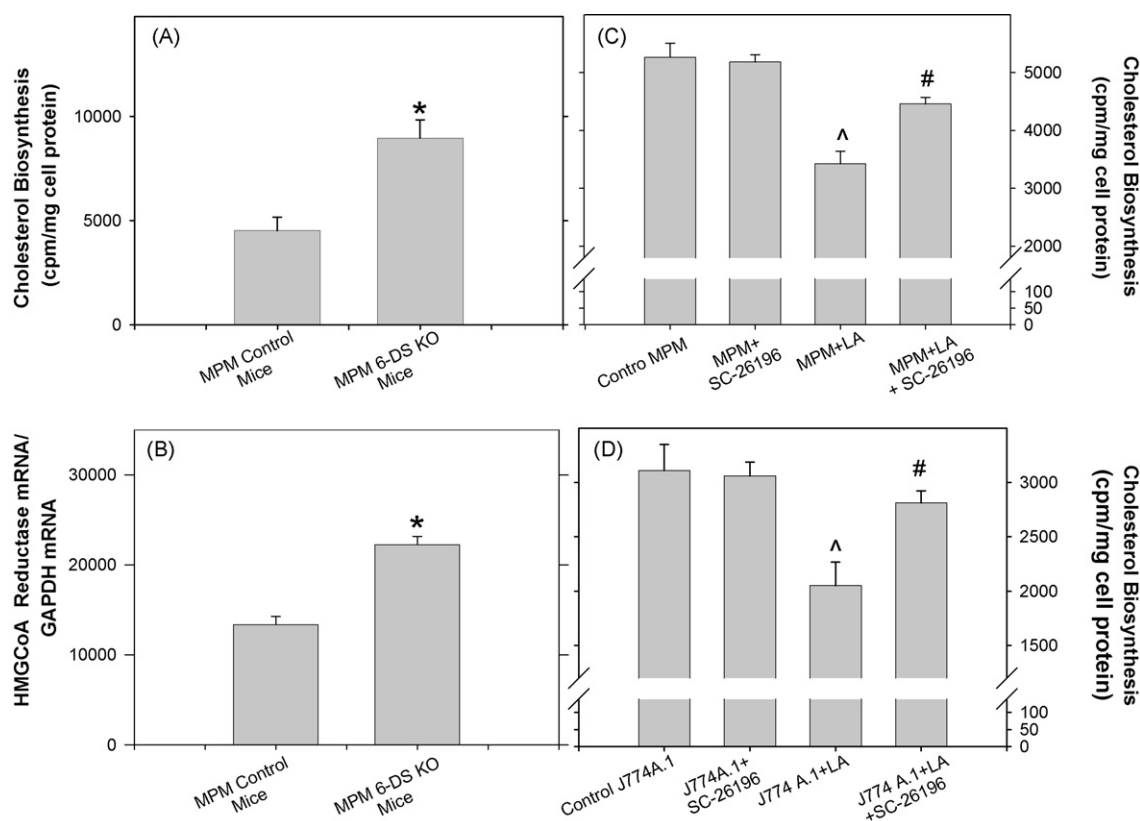


Fig. 1. Macrophage cholesterol biosynthesis in MPM from 6-DS KO mice and in cultured macrophages incubated with SC-26196. (A and B) MPM were harvested from 6-DS KO mice or from control (C57BL/6) mice. (A) Macrophage cholesterol biosynthesis rate. (B) Macrophage HMGCoA-reductase mRNA levels. MPM from control mice (C) or J774A.1 macrophage cell line (D) were incubated with no addition (control cells), with SC-26196 ($2 \mu\text{M}$), with LA ($2 \mu\text{M}$), or with LA ($2 \mu\text{M}$) + SC-26196 ($2 \mu\text{M}$). The extent of macrophage cholesterol biosynthesis was then determined. Results are given as mean \pm SD of three different experiments. * $p < 0.01$ vs. MPM control mice, ^ $p < 0.01$ vs. control cells, # $p < 0.01$ vs. cells + LA.

3.3.2. Macrophage PON2 expression in 6-DS KO mice and in cell cultured macrophages

PON2 lactonase activity (Fig. 2A) and PON2 mRNA levels (Fig. 2B) in the 6-DS KO vs. control mice MPM were significantly decreased, by 73% and 2.5-fold, respectively. Incubation of control mice MPM (Fig. 2C), or J774A.1 macrophages (Fig. 2D) with LA (2 μM), significantly increased PON2 lactonase activity by 42% or by 34%, respectively, as compared to control MPM or J774A.1 macrophages that were incubated with no addition, while incubation with SC-26196 did not affect PON2 lactonase activity in both types of macrophages (Fig. 2C and D).

In contrast, and similarly to the in vivo results, incubation of MPM or J744A.1 with LA together with SC-26196 completely abolished LA induced increment in PON2 activity (Fig. 2C and D).

3.3.3. Direct effect of AA on macrophage cholesterol biosynthesis rate: studies in MPM and in J774A.1 macrophages

We next questioned whether incubation of macrophages with AA can reverse the above atherogenic processes. For this purpose we have incubated macrophages (MPM or J774A.1) with AA (0–100 μM). These concentrations had no cytotoxic effects on the cells as measured by lactate dehydrogenase (LDH) release into the medium (14 ± 2 vs. 13 ± 1 units/mg cell protein in J774A.1 macrophages that were incubated with AA (100 μM) vs. control non-treated cells, respectively). Furthermore, the concentration of 100 μM resulted in only non-significant increase by 10% in cellular oxidative stress as measured by the DCFH analysis (88 ± 7 vs. 79 ± 6 mean fluorescence intensity). Incubation of control mice MPM with 50 μM or 100 μM of AA, significantly and dose-dependently decreased cellular cholesterol biosynthesis rate by 18% or by 43%, respectively (Fig. 3A), as compared to cells incubated only with medium. Similarly, in J774A.1 macrophages that

were incubated with AA (0–100 μM), cellular cholesterol biosynthesis rate substantially and dose-dependently decreased, by up to 74% (Fig. 3B). Furthermore, the cellular cholesterol content in these cells decreased in an AA dose-dependent manner, by up to 59% (Fig. 3C). These effects were specific for AA, since incubation of J774A.1 macrophages with 50 μM of DHA did not decrease the cellular cholesterol biosynthesis rate (3050 ± 210 in DHA-treated cells vs. 3108 ± 240 cpm/mg cell protein in the control cells).

3.3.4. Direct effect of AA on macrophage PON2 expression: studies in MPM and in J774A.1 macrophages

Incubation of control mice MPM with AA (50 μM), significantly increased cellular PON2 lactonase activity, by 32% (Fig. 4A), as compared to untreated cells. In parallel, PON2 mRNA levels in AA (50 μM or 100 μM)-treated MPM were significantly increased, by 33% and 70%, respectively, as compared to untreated cells (Fig. 4B). Similarly, incubation of J774A.1 macrophages with AA (50 μM or 100 μM) increased PON2 lactonase activity by 43% and 65%, respectively (Fig. 4C). These effects were specific for AA, since incubation of J774A.1 macrophages with 50 μM DHA did not increase the cellular PON2 lactonase activity, but even decreased it by 22% (9.5 ± 0.2 in DHA-treated cells vs. 12.2 ± 0.8 munits/mg cell protein in the control cells). AA was shown to activate AP-1 [27] and PPARγ [28], and activation of these transcription factors resulted in an increased macrophage PON2 expression [29]. Thus, we next analyzed the possible involvement of AP-1 and of PPARγ in AA-induced upregulation of PON2 expression. For this purpose we used the GW9662 (50 μM, specific irreversible inhibitor of PPARγ), and the SP600125 (20 μM, specific inhibitor of AP-1). Indeed, upon incubation of J774A.1 macrophages with AA (50 μM) together with the above inhibitors, there was a complete abolishment of the AA-induced increment (by 48%) in cellular PON2 lactonase activity (Fig. 4D).

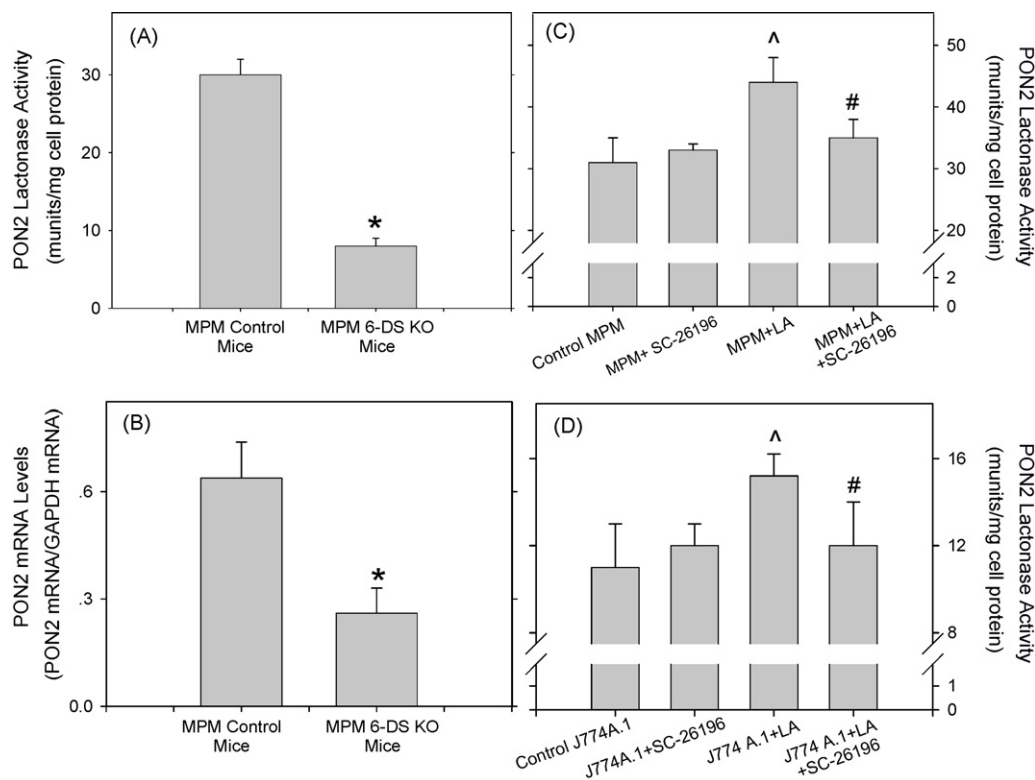


Fig. 2. Macrophage PON2 expression in 6-DS KO mice MPM and in cultured macrophages incubated with SC-26196. (A and B) MPM were harvested from 6-DS KO mice or from control (C57BL/6) mice. (A) Cellular PON2 lactonase activity and (B) MPM PON2 mRNA expression. MPM from control mice (C), or J774A.1 macrophage cell line (D), were incubated with no addition (control cells), with SC-26196 (2 μM), with LA (2 μM), or with LA (2 μM) + SC-26196 (2 μM). The cells were washed and cellular PON2 lactonase activity was determined. Results are given as mean ± SD of three different experiments. *p < 0.01 vs. MPM control mice, ^p < 0.01 vs. control cells, #p < 0.01 vs. cells + LA.

3.3.5. In vivo effect of AA consumption by 6-DS KO mice on MPM cholesterol biosynthesis rate and on PON2 expression

Finally, we questioned whether AA supplementation to 6-DS KO mice could act similarly to its action in vitro in a cell culture system (Figs. 3 and 4). For the sake of comparison, the control mice were also supplemented with AA.

In 6-DS KO mice MPM, cellular content of AA was substantially increased after AA supplementation (Fig. 5A). AA accumulation in MPM from the 6-DS KO mice was associated with a significant reduction, by 45% and 4-fold, in cellular cholesterol biosynthesis rate and in HMGCoA-reductase mRNA expression, respectively (Fig. 5B and C), as compared to 6-DS KO mice MPM that were fed with AIN diet. AA feeding of 6-DS KO mice significantly increased their MPM PON2 lactonase activity (Fig. 5D) and PON2 mRNA levels (Fig. 5E) by 44% and 2.3-fold, as compared to 6-DS KO mice fed with AIN diet. Control mice MPM contain significant amount of AA, but still, AA supplementation to these mice increased their MPM AA levels by 53% (from 2391 ± 100 to 3650 ± 150 ng/mg cell protein). However, the above further macrophage increment in AA following mice feeding with AA had no additional significant effect on MPM cholesterol biosynthesis rate and on PON2 expression in control mice (data not shown).

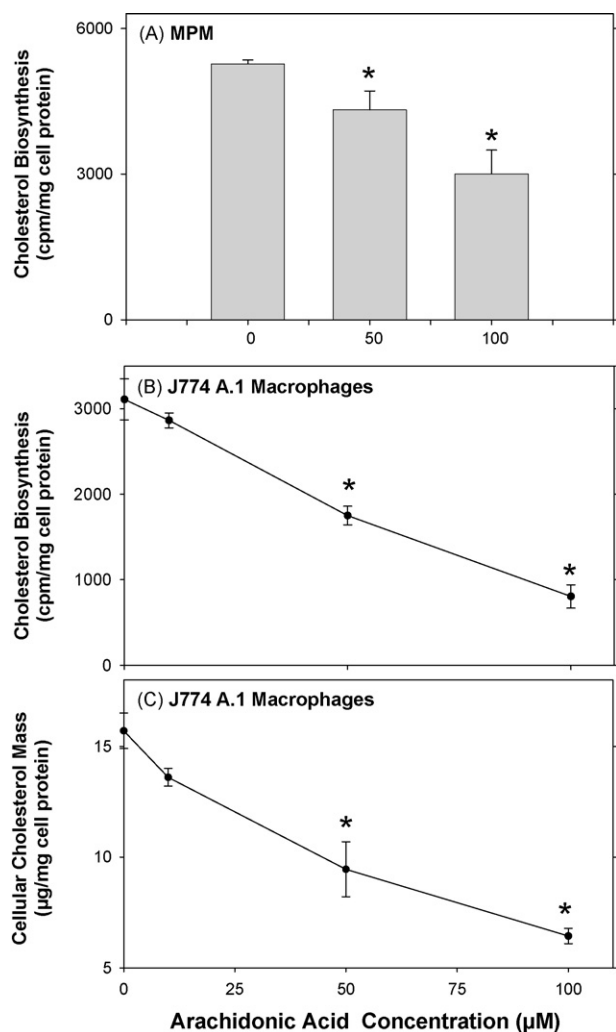


Fig. 3. Direct effect of AA on cellular cholesterol biosynthesis in MPM or in J774A.1 macrophages. MPM from C57BL/6 mice (A) or J774A.1 macrophage cell line (B and C) were incubated with increasing concentrations (0–100 μM) of AA. (A and B) Macrophage cholesterol biosynthesis rate. (C) Cellular cholesterol level. Results are given as mean \pm SD of three different experiments. * $p < 0.01$ vs. 0 concentration.

4. Discussion

In the current study we have demonstrated, for the first time, that macrophage arachidonic acid (AA) which is produced via the action of the rate-limiting enzyme $\Delta 6$ -desaturase (6-DS), possesses anti-atherogenic properties. AA inhibits HMGCoA-reductase mRNA expression and macrophage cholesterol biosynthesis, and upregulates cellular PON2 expression (mRNA and activity).

Our observations are based on studies that were performed in several systems: (a) MPM harvested from the 6-DS KO vs. control mice (receiving a diet with no AA). (b) MPM from 6-DS KO mice which received a diet that was supplemented with AA. (c) Cell cultured macrophages (control mice MPM or J774A.1) that were incubated with a 6-DS specific inhibitor, to mimic the situation present in 6-DS KO mice MPM. (d) Direct incubation of cell cultured macrophages with AA.

The enzyme 6-DS is expressed in many tissues [2], but data on its expression in macrophages are controversial. Using specific primers for 6-DS we have demonstrated, that MPM express 6-DS almost as much as kidney or liver does, whereas J774A.1 macrophage – like cell line had a significant lower expression than MPM does. Furthermore, we have demonstrated that MPM are capable of converting LA to AA, confirming the presence of active macrophage 6-DS. These latter results are in contrast with a previous study [30], probably due to methodology differences. We have used the J774A.1 macrophages, in addition to MPM, in order to demonstrate that the protective effects of AA are not unique to MPM, but probably relevant to all types of macrophages. Although the expression of 6-DS is much lower in J774A.1 macrophages than in MPM, we observed similar results in both types of macrophages, suggesting that low expression of 6-DS is enough to get the observed anti-atherogenic effects of AA.

To study the possible in vivo protective role of AA, we used the 6-DS KO mice model which allows us to assess the effect of AA deficiency, without the need to eliminate LA, the precursor of AA [14]. Indeed, in the 6-DS KO MPM, no AA could be detected. We have also used control (C57BL/6) mice MPM or J774A.1 macrophages that were incubated with the 6-DS specific inhibitor SC-26196 [26] to mimic the in vivo 6-DS deficiency situation. Indeed, this inhibitor significantly attenuated the conversion of LA to AA. In both the 6-DS KO mice MPM and in the SC-26196-treated macrophages (MPM, J774A.1) cellular cholesterol biosynthesis rate was significantly increased. In accordance with these results the macrophage HMGCoA-reductase mRNA levels were also significantly higher in the 6-DS KO vs. control MPM. These results suggest that AA has a direct effect on HMGCoA-reductase expression, and thus – on the extent of cellular cholesterol biosynthesis. Indeed, supplementation of AA to 6-DS KO mice increased the cellular AA content and decreased MPM HMGCoA-reductase mRNA level and cellular cholesterol biosynthesis rate. Similar results were observed upon direct incubation of macrophages (MPM or J774A.1) with AA. SREBPs were shown to activate cholesterol and/or fatty acid synthesis in the liver [31]. Whereas SREBP1-c does not affect cholesterol biosynthesis, it increases fatty acid synthesis, while SREBP-2 does not affect fatty acid synthesis, but increases cholesterol biosynthesis. SREBP1-a, in contrast, induces both cholesterol and fatty acids synthesis [31]. AA reduces SREBP-1a and -1c mRNA and protein expression in the nucleus whereas no such effect could be noted on SREBP2 in HEK-293 cells [32], rat hepatocytes [33] or rat liver [34], implicating that AA may suppress cholesterol biosynthesis by reducing SREBP-1a.

PON1 is an HDL-associated enzyme which was shown to protect lipoproteins, macrophages and atherosclerotic lesions against oxidative stress [13]. However, PON1 is not expressed in macrophages [13]. HDL-associated PON1 can internalizes the cells and affects their atherogenic properties (reduction in oxidative

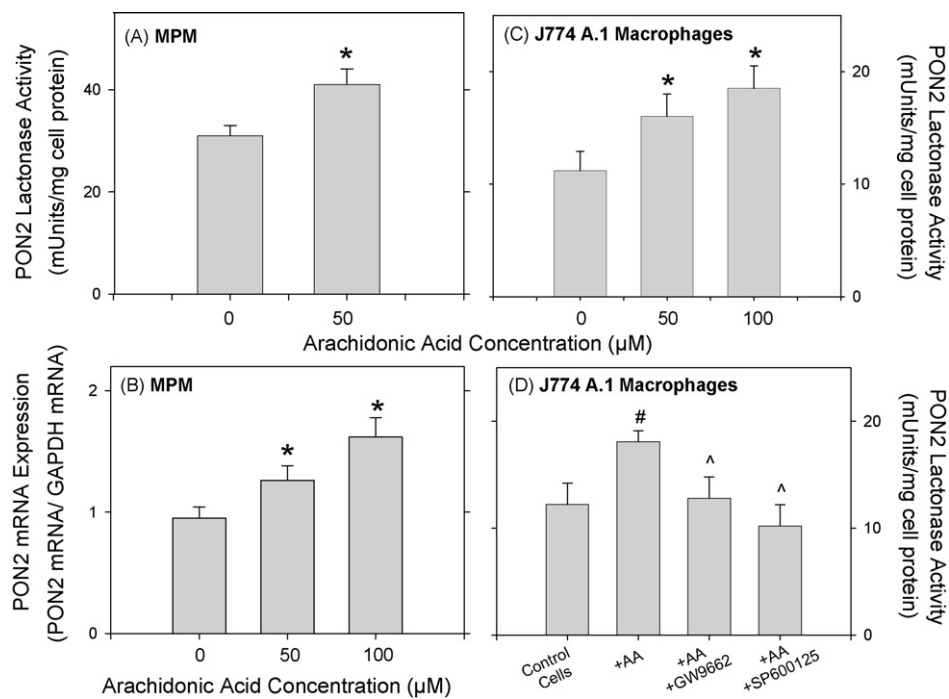


Fig. 4. Effect of AA on PON2 expression in MPM or in J774A.1 macrophages. MPM from C57BL/6 mice (A and B) or J774A.1 macrophage cell line (C) were incubated with increasing concentrations (0–100 μM) of AA. (A and C) PON2 lactonase activity. (B) PON2 mRNA levels. (D) J774A.1 macrophages were incubated for 20 h with no addition (control cells), with AA (50 μM), with AA (50 μM) + PPAR gamma inhibitor – GW9662 (50 μM), or with AA (50 μM) + AP-1 inhibitor – SP600125 (20 μM). The cells were then washed and PON2 lactonase activity was measured. Results are given as mean ± SD of three different experiments. **p* < 0.01 vs. 0 concentration, #*p* < 0.01 vs. control cells. ^*p* < 0.01 vs. +AA.

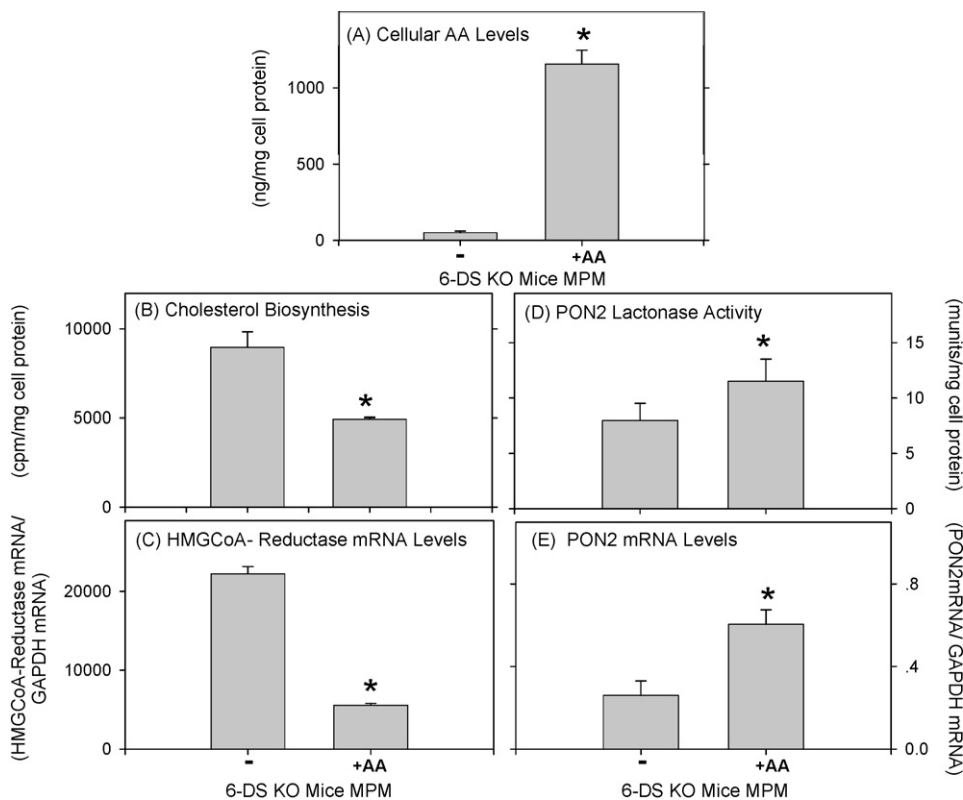


Fig. 5. Effect of AA supplementation to 6-DS KO mice on macrophage cholesterol biosynthesis, on cellular PON2 expression. 6-DS KO mice were supplemented with AIN diet (–), or with AIN diet +AA (+AA). At the end of the study, the mice MPM were harvested. (A) Cellular AA levels were measured by HPLC. (B) Macrophage cholesterol biosynthesis rate. (C) Macrophage HMGCoA-reductase mRNA expression. (D) Cellular PON2 lactonase activity. (E) Cellular PON2 mRNA levels. Results are given as mean ± SD of three different experiments. **p* < 0.01 vs. 6-DS KO mice MPM (–).

stress, inhibition of cholesterol biosynthesis, and stimulation of HDL-mediated cholesterol efflux from the cells) upon their incubation with HDL. By using PON1 mutants in the catalytic site, we were able to demonstrate that PON1 catalytic activities, and especially its lactonase activity, is required for its anti-atherogenic properties. In contrast to HDL-associated PON1, PON2 is cell-associated and it is expressed in macrophages [13]. PON2 was shown to protect the macrophages from oxidative stress and from triglyceride accumulation [15]. AA supplementation to 6-DS KO mice increased both serum PON1 activity (probably, via upregulation of PON1 expression in the liver) and macrophage PON2 expression. The increased macrophage cholesterol biosynthesis rate in the 6-DS KO mice could have possibly resulted also from the reduced serum PON1 activity, since PON1 was shown to inhibit macrophage cholesterol biosynthesis [35]. In contrast to PON1, PON2 does not inhibit macrophage cholesterol biosynthesis (it showed similar cholesterol biosynthesis rates in PON2 KO mice and in control mice).

We have previously demonstrated that cholesterol accumulation in macrophages is associated with decreased PON2 expression [18,19], similarly to the results observed in the current study in 6-DS KO macrophages, or in macrophages treated with the 6-DS inhibitor. These results suggest that AA may have a direct effect on macrophage PON2 expression. Indeed, supplementation of AA to 6-DS KO mice significantly increased their macrophage PON2 expression. Similar results were observed upon direct incubation of macrophages (MPM or J774A.1) with AA. In contrast to our results, a previous study [20] demonstrated upregulation of PON2 expression upon loading the cells with unesterified cholesterol. In that study however, the authors used an artificial system where J774A.1 macrophages were incubated with acetylated LDL together with the ACAT inhibitor 58035. It was previously shown that acetylated LDL loading of THP-1 macrophages could cause both intracellular unesterified cholesterol (UC) and cholesterol ester (CE) increases [36]. Addition of the ACAT inhibitor to the cells increased only the macrophage UC content and it was associated with increment of PON2 expression, in comparison to J774A.1 macrophages that were incubated with acetylated LDL without the inhibitor (increment in both UC and CE, 20). In MPM from 6-DS KO mice, as compared to MPM from control mice, there was a significant increase in total cholesterol content, which was associated with increment mostly in the macrophage CE levels. These results may suggest that macrophage CE accumulation might decrease PON2 expression, as was indeed previously shown [18,19]. Macrophage PON2 expression was previously shown to be upregulated via PPAR gamma and AP-1 pathway activation [29]. The increment in macrophage PON2 expression thus could probably result from AA-mediated activation of PPAR gamma [28], and/or of AP-1 [27]. Indeed, incubation of J774A.1 macrophages with AA in the presence of PPAR gamma or AP-1 specific inhibitors (GW9662 or SP600125, respectively) completely abolished the AA-induced increment in cellular PON2 lactonase activity. Upregulation of PON2 expression thus could be the result of a direct effect of AA, or via AA-induced reduction in cellular CE levels.

The observed effects on cholesterol biosynthesis and on PON2 expression in macrophages were specific for AA, since DHA at the same concentrations did not significantly affect macrophage cholesterol biosynthesis rate and in fact, it even decreased PON2 activity.

Supplementation of AA to control mice also increased the cellular AA levels in their MPM (that already contain AA), but no further significant effect could be noted on both their MPM cholesterol biosynthesis rate and PON2 expression, in comparison to macrophages from control mice fed with AIN diet lacking AA. It should be noted that AA supplementation to 6-DS KO mice increased the macrophage AA levels, but these levels were still much lower than those levels observed in control mice MPM.

Macrophage PON2 over expression was shown to attenuate atherosclerosis development [17]. The anti-atherosclerotic effect of macrophage PON2 was previously shown to be secondary to its ability to decrease macrophage oxidative stress, as well as, to inhibit cellular triglyceride accumulation and foam cell formation [13,15]. In accordance with the current study results it was recently demonstrated that accumulation of AA due to 5- or 12-lipoxygenase deficiencies, attenuated atherosclerosis development in the apoE KO mice model [37].

Additional research is needed to reveal the exact mechanism by which AA decreases macrophage cholesterol biosynthesis and increases PON2 expression. Intervention means to increase macrophage 6-DS activity (and AA formation) could probably contribute to attenuation of macrophage cholesterol accumulation and foam cell formation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2009.11.044.

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