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Macrophages from alpha 7 nicotinic acetylcholine receptor knockout mice demonstrate increased cholesterol accumulation and decreased cellular paraoxonase expression: A possible link between the nervous system and atherosclerosis development

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ABSTRACT

Objective: The parasympathetic nervous system regulates inflammation in peripheral tissues through a pathway termed the "cholinergic anti-inflammatory reflex" (CAIR). Mice deficient in the alpha 7 nicotinic acetylcholine receptor ($\alpha7^{-/-}$) have an impaired CAIR due to decreased signaling through this pathway. The purpose of this study was to determine if the increased inflammation in $\alpha7^{-/-}$ mice is associated with enhanced serum and macrophage atherogenicity.

Methods: We measured serum markers of inflammation and oxidative stress, and macrophage atherogenicity in mouse peritoneal macrophages harvested from $\alpha7^{-/-}$ mice on the background of C57BL/6 mice, as well as on the background of the atherosclerotic Apolipoprotein E-deficient ($ApoE^{-/-}$) mice.

Results: α7-Deficiency had no significant effects on serum cholesterol, or on markers of serum oxidative stress (TBARS and paraoxonase1 activities). However, α7-deficiency significantly increased serum CRP and IL-6 ($p < 0.05$) levels in atherosclerotic mice, confirming an anti–inflammatory role for the α7 receptor. Macrophage cholesterol mass was increased by 25% in both normal and atherosclerotic mice in the absence of the α7 receptor ($p < 0.05$). This was accompanied by conditional increases in oxidized LDL uptake and in macrophage total peroxide levels. Furthermore, α7-deficiency reduced macrophage paraoxonase2 mRNA and activity by 50–100% in normal and atherosclerotic mice ($p < 0.05$ for each), indicating a reduction in macrophage anti-oxidant capacity in the $\alpha7^{-/-}$ mice.

Conclusion: The above results suggest an anti–atherogenic role for the macrophage α7nAchr, through a mechanism that involves attenuated macrophage oxidative stress and decreased uptake of oxidized LDL.

Introduction

Recently, Tracey et al. discovered a novel neural pathway that regulates inflammation in peripheral tissues termed the "cholinergic anti-inflammatory reflex" (CAIR) [1]. This reflex describes a pathway by which pro-inflammatory cytokines produced in tissues bind to and activate receptors on afferent parasympathetic (vagal) nerves, eliciting a central nervous system response to the inflammation. Subsequent efferent vagal activity leads to acetylcholine release in the inflamed tissues. Resident tissue macrophages express the alpha 7 nicotinic acetylcholine receptor (α7nAchr). Activation of this receptor inhibits the production of pro-inflammatory cytokines, thereby attenuating the local inflammatory response [2]. Mice deficient in the α7nAchr ($\alpha7^{-/-}$) have an impaired CAIR, leading to an exaggerated response to inflammatory stimuli [1]. In contrast, pharmacological activation of the α7nAchr has been shown to inhibit inflammation [1].

Atherosclerosis is an inflammatory disease typically characterized by the influx of macrophages and other immune cells into the artery wall from the vessel lumen [3]. Recent studies also show that pro-inflammatory cytokine production by resident macrophages in the outer layers of the artery wall (adventitia) and surrounding adipose tissue (peri-adventitia) may play a significant role in the pathogenesis of atherosclerosis [4]. Vagal nerve fibers innervate

Abbreviations: α7nAchr, alpha 7 nicotinic acetylcholine receptor; AAPH, 2.2′-azobis, 2-amidinopropane hydrochloride; CAIR, cholinergic anti-inflammatory reflex; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; FITC, fluoroisothiocyanate; MFI, mean fluorescence intensity; MLA, methyllycaconitine; MPM, mouse peritoneal macrophage; PMA, phorbol myristate acetate; PON, paraoxonase; TBARS, thiobarbituric acid reactive substances; TBBL, 5-thiobuthyl-butyrolactone

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the adventitial layer of both the coronary arteries and aorta [5,6]; however, no studies have examined if the CAIR contributes to the regulation of arterial inflammation and/or atherosclerosis.

The purpose of the current study was to assess the potential role of the CAIR in macrophage atherogenicity, a hallmark of early atherogenesis, by examining systemic markers of inflammation and oxidative stress, as well as the atherogenicity of macrophages harvested from $\alpha7^{-/-}$ mice. Macrophage atherogenic parameters included cellular cholesterol content, lipoprotein uptake, and oxidative stress, as well as the expression of paraoxonase 2 (PON2), a cellular anti-oxidant and anti- atherosclerotic enzyme [7]. We hypothesized that $\alpha7^{-/-}$ mice would have increased circulating markers of inflammation and oxidation, increased macrophage cholesterol accumulation and oxidative stress, and decreased macrophage PON2 expression.

Materials and methods

Animals. Four groups of mice were used for these experiments: (1) $\alpha7^{+/+}$/ApoE$^{+/+}$ (wild type), (2) $\alpha7^{-/-}$/ApoE$^{+/+}$, (3) $\alpha7^{+/+}$/ApoE$^{-/-}$, and (4) $\alpha7^{-/-}$/ApoE$^{-/-}$ ($n = 3$ male mice/group). These mice were produced from a breeding colony that was started by crossing $\alpha7^{-/-}$ mice on a C57BL/6J background (B6.129S7-Chrna7$^{tm1Bay/J}$; Jackson Laboratories; # 003232) with ApoE$^{-/-}$ mice on a C57BL/6 J background (B6.129S7-ApoE$^{tm1Unc/J}$; Jackson Laboratories; # 002052). All mice were housed in plastic cages in temperature-controlled rooms (22 °C) with a 12-h light/12-h dark cycle, weaned at 4 weeks, and placed on a standard chow diet until sacrificed at 3 months of age. NIH guidelines for the care and use of laboratory animals were strictly followed and all experiments were approved by the Animal Care and Use Committee at the University of Illinois.

Blood analysis. Blood was taken at sacrifice by retro-orbital bleeding and serum collected by centrifugation. Serum total cholesterol levels were measured using a commercial enzymatic kit (Wako Inc., Richmond, VA). Serum CRP and IL-6 levels were determined using commercially available high sensitivity Elisa kits (R&D Systems, MN, USA).

Serum lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay, in the presence and absence of the free radical generator 2.2′-azobis, 2-amidinopropane hydrochloride (AAPH), as described [8]. Serum paraoxonase 1 (PON1) arylesterase activity was measured to assess serum anti-oxidant capacity using phenylacetate as the substrate, as described [9].

Mouse peritoneal macrophages (MPM). MPM were harvested from each animal 4 days after intraperitoneal injection of 3 ml thioglycolate (40 g/L) in saline. The cells (10–20 $\times$ 10^6/mouse) were washed three times with phosphate-buffered saline (PBS) at 1000*g* for 10 min, then resuspended at 10^9/L in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% FCS. The dishes were incubated for 2 h at 5% CO_2, washed with DMEM to remove nonadherent cells, then used for the analyses described below.

Macrophage total peroxides. Intracellular oxidative stress was assayed by the oxidation of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) [10], as monitored by flow cytometry. MPMs were incubated with 10 μmol/L of DCFH-DA, for 30 min at 37 °C. Adherent cells were detached by scraping, and cells were washed twice with PBS. Cellular fluorescence determined by FACS was performed at 510–540 nm after excitation of the cells at 488 nm with an argon ion laser. Cellular fluorescence was measured in terms of mean fluorescence intensity (MFI).

LDL and oxidized LDL (Ox-LDL) uptake by macrophages. LDL was separated from plasma of healthy volunteers by discontinuous density-gradient ultracentrifugation, then dialyzed, as described [11]. Oxidation of LDL was carried out at 37 °C in a water bath. LDL (1 mg/ml) was incubated for 18 h at 37 °C with 5 μmol/L freshly prepared $CuSO_4$. The extent of LDL oxidation was determined by the TBARS assay [8]. LDL and Ox-LDL were conjugated to fluoroisothiocyanate (FITC) for cellular uptake studies [12]. MPM were incubated at 37 °C for 3 h with FITC-conjugated LDL or Ox-LDL at a final concentration of 25 μg of protein/ml. The uptake of the lipoprotein was determined by flow cytometry. Measurements of cellular fluorescence determined by FACS were done at 510 nm to 540 nm after excitation of the cells at 488 nm with an argon ion laser. Cellular fluorescence was measured in terms of mean fluorescence intensity (MFI).

Macrophage–mediated LDL oxidation. Human LDL (after dialysis) was incubated (100 μg of protein/mL) with no cells, or with MPM (2×10^6) in the presence of 5 μmol/L $CuSO_4$ for 3 h at 37 °C, and the extent of cell-mediated LDL oxidation was determined in the culture medium by the TBARS assay [8].

Superoxide anion generation by macrophages. The production of the superoxide anion ($O_2^{\cdot-}$) by MPM was measured as the superoxide dismutase inhibitable reduction of acetyl ferricytochrome *C*, as described [13]. Superoxide anion production by the cells was stimulated by the addition of phorbol myristate acetate (PMA,100 ng/mL). The reduction of acetyl ferricytochrome *C* was followed by the changes in absorbance at 550 nm after 1 h incubation at 37 °C. The amount of superoxide anion released from the cells was expressed as nmol of superoxide per mg cell protein using an extinction coefficient of 21(mmol/L)$^{-1}$ cm^{-1} [13].

Macrophage cholesterol mass. Lipids from the MPM (2×10^6) were extracted with hexane: isopropanol (3:2, v:v) and the hexane phase was evaporated under nitrogen. The amount of cellular cholesterol was determined using a commercially available kit (Roche Diagnostics GMbH, Germany).

Macrophage PON2 lactonase activity. MPMs were seeded in a 96-well plate (2×10^5 per well). Initial rates of hydrolysis were determined spectrophotometrically at 412 nm for 3 min. The assay mixture included 0.25 mmol/L 5-thiobuthyl-butyrolactone (TBBL) and 1 mmol/L $CaCl_2$ in 50 mmol/L Tris–HCl, pH 8.0. Non-enzymatic hydrolysis of TBBL was subtracted from the total rate of hydrolysis. One unit of lactonase activity is equivalent to 1 μmol of TBBL hydrolyzed/min/mL [14]. The cells were then washed and 0.1 N NaOH was added for protein determination by the Lowry assay[12].

To examine the effect of methyllycaconitine (MLA), a specific inhibitor of the α7nAchr [15], on PON2 expression (activity, mRNA), macrophages from control ($\alpha7^{+/+}$/ApoE$^{+/+}$) mice were seeded as described above, then incubated for 20 h at 37 °C with 0, 1, 10, or 100 nmol/L MLA.

mRNA expression of CD36, CRP, and PON2. Total RNA was extracted from cells with Epicentre commercial kit (Tamar, Israel), and cDNA was generated from using Thermo Scientific commercial kit (Tamar, Israel). Products of the RT were subjected to Quantitative PCR using TaqMan Gene Expression Assays on the Rotor-Gene 6000 system (Corbett Life Science, Australia). Expression of genes of interest were normalized to the expression of GAPDH mRNA. The primers used are listed below:

CD36: sense, 5′-TGCGAACTGTGGGCTCATTG-3′; antisense, 5′-CC TCGGGGTCCTGAG TTATATTTT C-3′.

PON2: sense, 5′-CGACTTAAAGCCTCCAGAGAA-3′; antisense, 5′-GG AATTTTAGACCCA CACTAAA.

CRP: sense, 5′-CATGTCTAAACAGGCCTTCG-3′; antisense, 5′-GCCA CTACACAAATA GTTCGG-3′.

GAPDH: sense, 5′-CTGCCATTTGCAGTGGCAAAGTGG-3′; antisense, 5′-TTGTCATGG ATGACCTTGGCCAGG-3′.

Statistics. Independent samples *t*-tests were used to compare differences in measured variables between α7nAchr genotype groups ($\alpha7^{-/-}$ and $\alpha7^{+/+}$) within the same ApoE genotype (ApoE$^{+/+}$ or ApoE$^{-/-}$). ANOVAs comparing all four genotype groups were not conducted because the ApoE$^{+/+}$ and ApoE$^{-/-}$ mice were not littermates and the experiments on these animals were not conducted simultaneously, so direct comparisons between these

groups is not appropriate. All data are expressed as means ± SD of three different experiments. A p value of <0.05 was considered as statistically significant.

Results

Serum cholesterol, paraoxonase1 activity, oxidative stress, and inflammatory parameters

Absence of the α7nAchr in mice ($\alpha7^{-/-}$) had no effect on serum cholesterol concentration, on AAPH-induced serum lipid peroxidation, or on the anti-oxidant enzyme PON1 activity in either the $ApoE^{+/+}$ or $ApoE^{-/-}$ mice (Fig. 1A–C). There was also no significant difference in serum CRP and IL-6 between $a7^{-/-}/ApoE^{+/+}$ and $a7^{+/+}/ApoE^{+/+}$ mice (Fig. 1D–E). However, in $ApoE^{-/-}$ mice, serum CRP and IL-6 levels were significantly ($p < 0.01$) increased in the absence of the α7nAchr by 89% or by 120%, respectively, compared to $\alpha7^{+/+}/ApoE^{-/-}$ mice (Fig. 1D–E). These results suggest that the α7nAchr helps reduce systemic inflammation which is present in the atherosclerotic $ApoE^{-/-}$ mice, but much less so in the Apo $E^{+/+}$ mice.

Macrophage atherogenicity in mice lacking the α7nAchr

Inflammation – CRP expression

Analysis of MPM CRP mRNA levels revealed a significant ($p < 0.05$) 1.9-fold increase in the $\alpha7^{-/-}/ApoE^{+/+}$ mice compared to $\alpha7^{+/+}/ApoE^{+/+}$ mice (1.15 ± 0.06 vs 0.59 ± 0.03 CRP/GAPDH ratio, respectively). In contrast, there was no significant difference in MPM CRP expression, between $\alpha7^{-/-}/ApoE^{-/-}$ mice compared to $\alpha7^{+/+}/ApoE^{-/-}$ mice (1.14 ± 0.06 vs 1.05 ± 0.05 CRP/GAPDH ratio, respectively).

Cholesterol metabolism

In both $ApoE^{+/+}$ or $ApoE^{-/-}$ mice, the absence of the α7nAchr significantly increased macrophage cholesterol mass by 29% or by 21%, respectively (Fig. 2A; $p < 0.05$ for both). The above phenomenon could have resulted from increased macrophage lipoprotein (LDL) uptake. Indeed, in $ApoE^{+/+}$ macrophages, the absence of α7nAchr significantly increased oxidized LDL (Ox-LDL) uptake by 35% (Fig. 2B, $p < 0.05$), whereas native LDL uptake by MPM decreased in the absence of α7nAchr by 26% (Fig. 2C, $p < 0.05$). In accordance with these results, CD36 mRNA levels were significantly ($p < 0.05$) increased by 1.9-fold in the $\alpha7^{-/-}/ApoE^{+/+}$ vs. $\alpha7^{+/+}/ApoE^{+/+}$ mice (data not shown).

In contrast to those observations in $ApoE^{+/+}$ macrophages, in the atherosclerotic $ApoE^{-/-}$ mice, who are under high oxidative stress, the absence of the α7nAchr was associated with a significant ($p < 0.05$) 35% increase in native LDL uptake (Fig. 2C), but did not significantly affect oxidized LDL uptake (Fig. 2B).

Cellular oxidative stress

The increased cellular uptake of native LDL by macrophages from the $ApoE^{-/-}$ mice could possibly be related to the increased cellular oxidative stress in those atherosclerotic mice. Indeed, there was a significant ($p < 0.05$) 32% increase in MPM total peroxide content in $\alpha7^{-/-}/ApoE^{-/-}$ mice compared to $\alpha7^{+/+}/ApoE^{-/-}$ mice (Fig. 2D), as well as a significant 35% increase in MPM superoxide anion release from $\alpha7^{-/-}/ApoE^{-/-}$ MPM compared to $\alpha7^{+/+}/ApoE^{-/-}$ MPM (23.3 ± 2.1 vs. 17.3 ± 0.9 nmol/mg cell protein, $p < 0.05$).

Different results were obtained in the control $ApoE^{+/+}$ mice as there was a significant ($p < 0.05$) 23% decrease in MPM total peroxide content in $\alpha7^{-/-}/ApoE^{+/+}$ mice compared to $\alpha7^{+/+}/ApoE^{+/+}$ mice (Fig. 2D), as well as a significant ($p < 0.05$) 28% decrease in cell–mediated LDL oxidation that were observed on using $\alpha7^{-/-}/ApoE^{+/+}$ MPM compared to $\alpha7^{+/+}/ApoE^{+/+}$ MPM (359 ± 35 vs 502 ± 9 nmol TBARS/mg LDL protein/mg cell protein; $p < 0.05$).

Analysis of LDL oxidation during macrophage incubation with the lipoprotein in the above systems revealed that under incubation of native LDL with MPM from the $ApoE^{-/-}$, the native LDL became oxidized. This was indicated by a significant increase in TBARS formation in the culture medium, which increased from 5 ± 2 to 33 ± 6 nmol TBARS/mg LDL protein, $p < 0.05$). There was

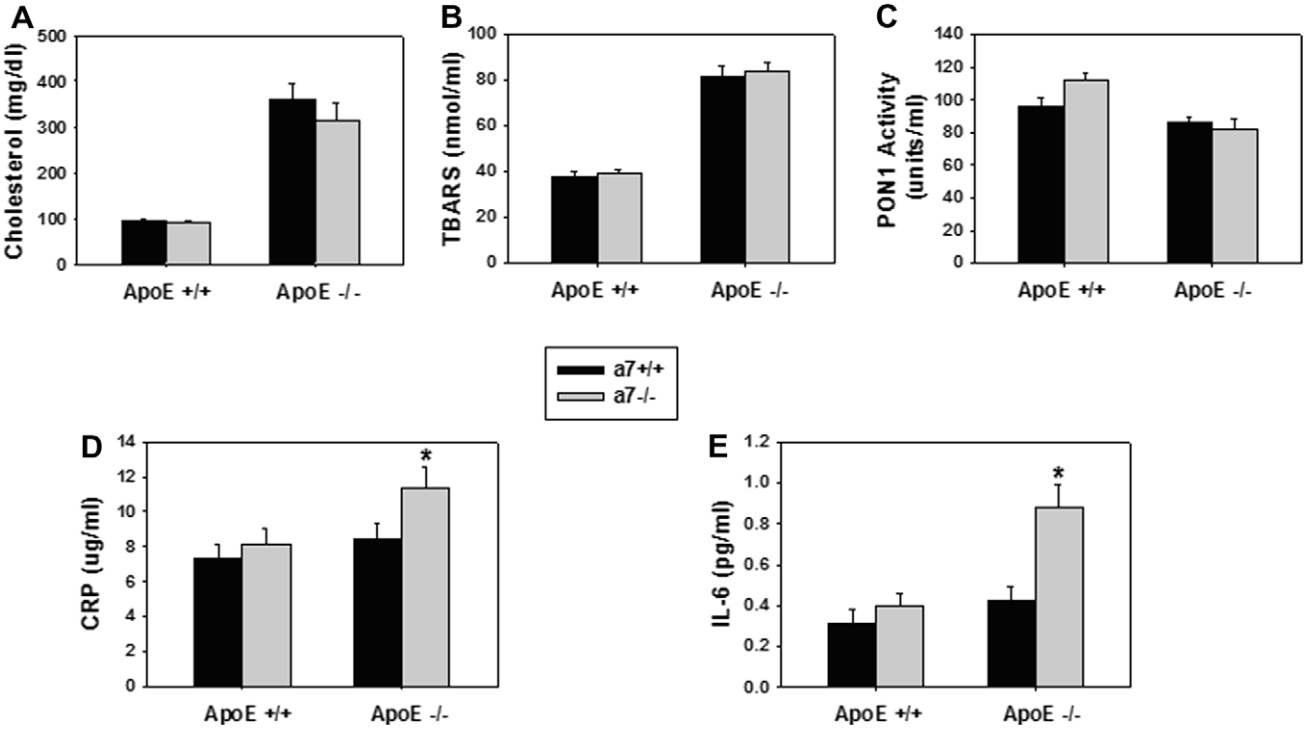

Fig. 1. Serum parameters. Blood was collected from all mice groups at sacrifice to measure serum total cholesterol (A), AAPH-induced lipid peroxidation (B), PON1 activity (C), CRP (D), and IL-6 (E).

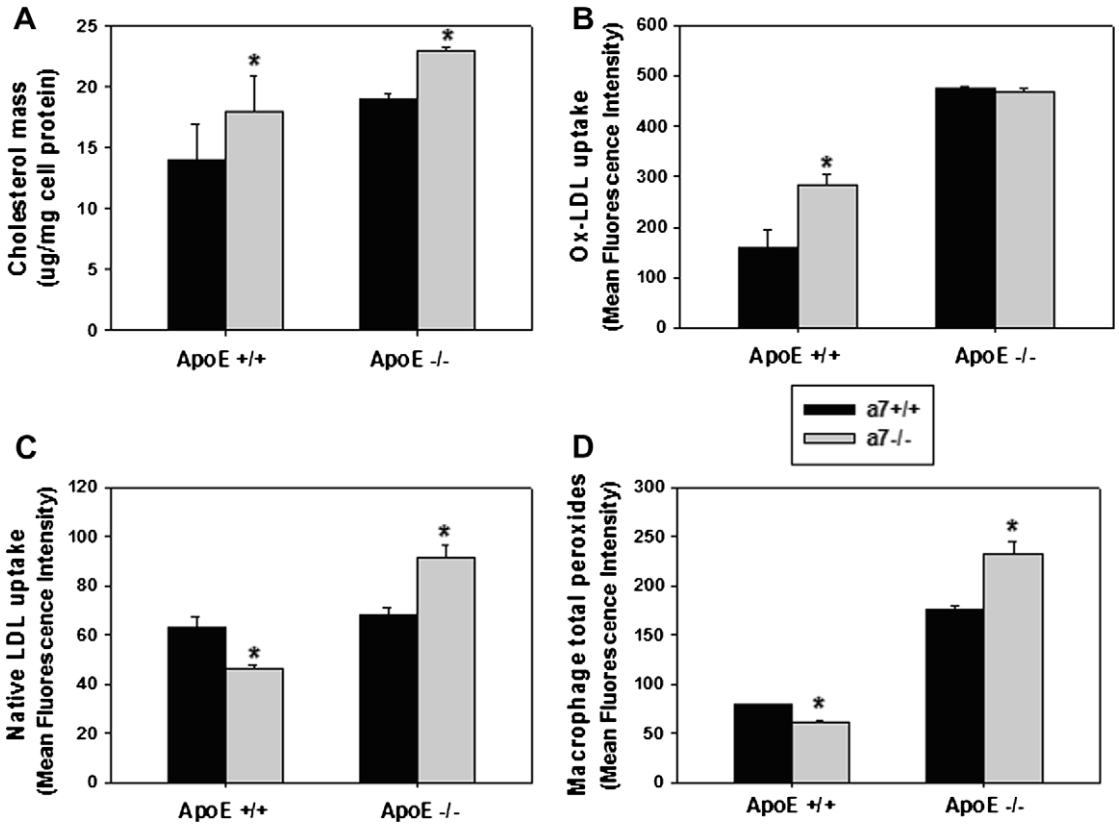

Fig. 2. Macrophage atherogenic parameters. Macrophages were harvested from the peritoneal fluid of all mice groups after thioglycolate injection. Macrophage cholesterol content (A), native LDL uptake (B), Ox-LDL uptake (C), and macrophage total peroxide levels (D).

no significant effect however on lipoprotein oxidation in the culture medium when MPMs from ApoE$^{+/+}$ mice were incubated with either native or oxidized LDL, or when MPMs from ApoE$^{-/-}$ mice were incubated with oxidized LDL (data not shown).

Cellular PON2 expression

Finally, as macrophage cholesterol accumulation and cellular oxidation has been shown to affect the expression of the cellular anti-oxidant/anti-atherogenic enzyme PON2 [16], we next measured MPM PON2 lactonase activity and mRNA expression. Absence of the α7nAchr significantly ($p < 0.01$) and substantially reduced PON2 expression in both ApoE$^{+/+}$ or ApoE$^{-/-}$ mice. This was indicated by an 85% reduction in both MPM PON2 lactonase activity (Fig. 3A) and PON2 mRNA expression (Fig. 3B) in the α7$^{-/-}$/ApoE$^{+/+}$ mice compared to α7$^{+/+}$/ApoE$^{+/+}$ mice. Likewise, MPM PON2 activity (Fig. 3A) and mRNA expression (Fig. 3B) were reduced by 48% and 97%, respectively, in the α7$^{-/-}$/ApoE$^{-/-}$ mice compared to α7$^{+/+}$/ApoE$^{-/-}$ mice.

Similar results were observed upon MPM incubation with MLA, a specific inhibitor of the macrophage α7nAchr. PON2 activity was decreased by 38% or 64% in the presence of 10 or 100 nmol/L of MLA, respectively (Fig. 3C). Likewise, PON2 mRNA expression was also decreased by 32% or by 27% ($p < 0.05$) in the presence of 10 nmol/L or 100 nmol/L of MLA, respectively (Fig. 3D).

Discussion

The primary findings in the present study are that the absence of the α7nAchr in mice is pro-inflammatory and pro–atherogenic, as it resulted in several significant effects: (1) increased systemic markers of inflammation (serum CRP and IL-6); (2) increased macrophage oxidative stress; (3) enhanced macrophage uptake of LDL (oxidized and/or native) and its consequent cholesterol accumulation; and (4) decreased macrophage anti-oxidant capacity by reducing cellular PON2 expression. These results clearly suggest an anti-atherogenic role for the macrophage α7nAchr through mechanisms that involve attenuated macrophage uptake of oxidized LDL and enhanced macrophage PON2 expression. Furthermore, these findings suggest a novel mechanism by which the central nervous system could potentially influence atherogenesis, secondary to the inhibition of vascular inflammation through the CAIR (see proposed model, Fig. 4).

Previous studies have shown that α7$^{-/-}$ mice have an exacerbated response to inflammatory stimuli [1]. Our finding that α7nAchr deficiency increases serum CRP and IL-6 in the ApoE$^{-/-}$ mice indeed supports an anti-inflammatory role for this receptor. The increase in systemic inflammation occurred despite no significant differences between α7$^{-/-}$ and α7$^{+/+}$ mice in serum cholesterol levels, serum markers of oxidative stress, or serum anti-oxidant capacity.

The effects of the α7nAchr on macrophage oxidative stress were more complex and somewhat dependent on the presence or absence of the ApoE gene. In ApoE$^{-/-}$ mice, MPM oxidative stress was significantly increased in the absence of the α7nAchr, whereas in ApoE$^{+/+}$ mice, MPM oxidative stress was somewhat decreased in the absence of the α7nAchr. These contradictory findings may be due to the well recognized high macrophage oxidative stress which is present in ApoE$^{-/-}$ mice [17], as indicated by their 2-fold higher total peroxides levels compared to ApoE$^{+/+}$ MPM (Fig. 2D). Thus, we hypothesize that when oxidative stress is low (e.g., in ApoE$^{+/+}$ mice), the α7nAchr may promote a modest, not harmful, increase in oxidative stress. While excessive oxidation is generally regarded as harmful, modest oxidative stress may be beneficial, as modest oxidative stress is necessary for normal cellular functions and even

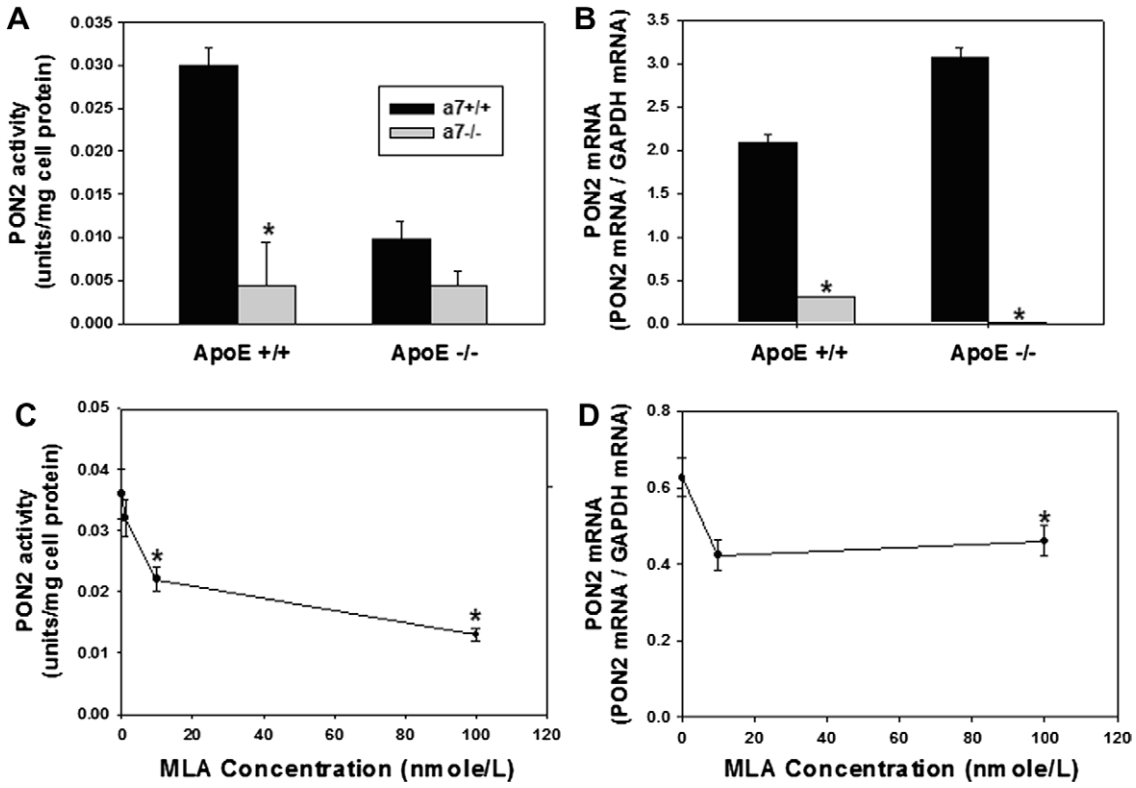

Fig. 3. Macrophage PON2 expression. PON2 lactonase activity (A) and mRNA levels (B) were measured in peritoneal macrophages harvested from all mice groups. (C&D) MPM from $\alpha7^{+/+}$/ApoE$^{+/+}$ mice were incubated for 20 h with increasing concentrations (0–100 nmol/L) of MLA. After cell wash, PON2 lactonase activity (C), and PON2 mRNA levels (D) were determined.

critical for normal immune functions [18]. On the contrary, under high oxidative stress (e.g., in ApoE$^{-/-}$ mice), we expect that the suggested α7nAchr-associated reductions in oxidative stress could clearly be atheroprotective. Additional studies will be needed however to determine the exact mechanisms responsible for the associations between the α7nAchr and macrophage oxidative stress.

Macrophage PON2 protects against atherosclerosis development, as it is a cellular (and not humoral) anti-oxidant enzyme that can hydrolyze some specific macrophage oxidized lipids [7,16]. The regulation of macrophage PON2 is complex; PON2 overexpression decreases oxidative stress [16], but on the other hand, macrophage PON2 is upregulated by modest oxidative stress (as a compensatory mechanism), via an NADPH oxidase-related mechanism [13,19]. In both ApoE$^{+/+}$ and ApoE$^{-/-}$ mice, absence of the α7nAchr significantly reduced MPM PON2 expression. To confirm the *ex- vivo* effect on macrophage PON2, we incubated MPMs from wild type mice ($\alpha7^{+/+}$/ApoE$^{+/+}$) in the absence or presence of MLA, a specific α7nAchr inhibitor. Previous research has shown that macrophages express and secrete their own acetylcholine, which mediates cellular functions in an autocrine and paracrine manner [20]. We thus hypothesized that MLA would block the autocrine activation of the α7nAchr, thereby reducing macrophage PON2 activity and expression. Our finding that PON2 mRNA and activity were reduced by MLA confirms our *ex-vivo* suggestion that the α7nAchr activates PON2.

It is well established that CRP is an acute phase protein released from hepatocytes that may contribute to the pathogenesis of various inflammatory diseases [21]. In addition, CRP is also expressed in macrophages [22]. Macrophage CRP mRNA levels were increased in the present study by 90% in MPMs from $\alpha7^{-/-}$/ApoE$^{+/+}$ mice compared to in $\alpha7^{+/+}$/ApoE$^{+/+}$, suggesting that the α7nAchr normally dampens macrophage inflammation [1]. However, we did not see a significant effect of the α7nAchr on CRP mRNA levels in MPMs harvested from the atherosclerotic ApoE$^{-/-}$ mice. We hypothesize that this may be because the excessive inflammation in the ApoE$^{-/-}$ mice is too extreme to be overcome by the modest anti-inflammatory effects of the α7nAchr.

Of special interest to α7nAchr anti–atherogenicity is the finding that absence of the α7nAchr increased macrophage cholesterol mass in both ApoE$^{+/+}$ and ApoE$^{-/-}$ mice. In ApoE$^{+/+}$ mice, this was associated with a significant increase in oxidized LDL uptake, a hallmark of early atherosclerosis development [23]. Furthermore, there was a 90% increase in MPM CD36 mRNA expression, a scavenger receptor primarily responsible for Ox-LDL uptake by macrophages [24]. The increase in CD36 expression indeed suggests a potential mechanism for the increased oxidized LDL uptake by these mice. By contrast, in the atherosclerotic ApoE$^{-/-}$ mice (that are under high oxidative stress), absence of the α7nAchr increased the macrophage uptake of "native" LDL, but not that of "oxidized" LDL. While these results appear to be paradoxical, the discrepancy may be explained by peculiarities of the assays used to measure "native" and "oxidized" LDL uptake. Previous research has shown that when native LDL is incubated in the presence of MPM from ApoE$^{-/-}$ mice (which are "oxidized"), a significant percentage of the LDL is oxidized in the culture medium prior to being taken up by the macrophages [17]. Indeed, when we incubated MPM from ApoE$^{-/-}$ mice with "native" LDL, LDL oxidation, as measured by TBARS levels in the culture medium, increased by 9-fold. However, when we incubated MPM from ApoE$^{-/-}$ mice with "oxidized" LDL, there was no additional increase in the Ox-LDL TBARS content. This suggests that the increased "native" LDL uptake in the $\alpha7^{-/-}$/ApoE$^{-/-}$ mice may actually have been due to an increase in the uptake of native LDL that had been oxidized in the culture medium by the oxidized cells, prior to being taken up by the macrophages.

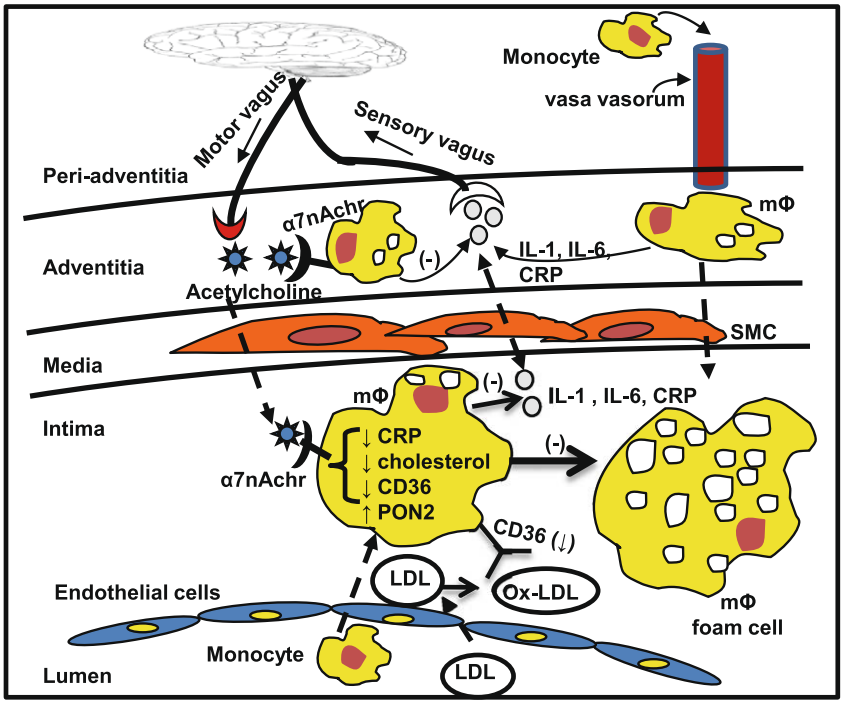

Fig. 4. Proposed model illustrating the possible role of the α7nAchr and the cholinergic anti-inflammatory reflex in atherosclerosis. Inflammatory stimuli promote macrophage accumulation in both the intima (from the systemic circulation) and adventitia (from the vasa vasorum) of the artery wall. The vagus nerve innervates the adventitial layer of epicardial coronary arteries and the aorta. Inflammatory cytokines produced by arterial wall macrophages may bind and activate afferent vagal fibers in the adventitia. Subsequent activation of efferent vagal fibers results in the release of acetylcholine (Ach) in the adventitia. Ach can bind to α7nAchr on adventitial macrophages, or diffuse across the media to the intima where it can bind to intimal macrophage α7nAchr and inhibit inflammatory cytokine production (IL-1, IL-6, CRP) by these cells, reduce CD36 expression, reduce macrophage cholesterol content, and increase PON2 expression. The resulting decrease in inflammatory cytokine production and cholesterol uptake by macrophages will inhibit foam cell formation, and hence, atherosclerosis. In the absence of the α7nAchr, inflammatory cytokine production by intimal or adventitial macrophages will persist, thus promoting atherosclerosis. Dashed arrows represent movement of cells, cytokines, proteins, or neurotransmitters between the different layers of the artery wall during atherogenesis.

Most importantly, the increase in MPM cholesterol mass in the absence of the α7nAchr in both ApoE$^{-/-}$ and ApoE$^{+/+}$ mice clearly suggests an anti-atherogenic role for the a7nAchr against macrophage cholesterol accumulation and foam cell formation.

Finally, several limitations to the present study should be considered. First, the experiments were conducted in a small number of animals, and even though all mice in each group demonstrated a very similar atherogenic pattern, we may have lacked statistical power to detect differences in some of our outcome variables. Second, while the changes we observed in macrophage phenotype suggest that the α7nAchr has an atheroprotective function, this needs to be directly tested by measuring atherosclerosis lesion size in a larger group of animals, which we are currently planning. Third, additional studies are needed to assess mechanisms for the potentially atheroprotective effects that were noted, particularly to determine if the differences in macrophage phenotype are directly mediated by the α7nAchr, or indirectly mediated via its well established effects on macrophage inflammatory cytokine production [1].

Conclusions

In summary, we demonstrated that a deficiency in the macrophage α7nAchr in mice increases markers of systemic inflammation. Most importantly, α7nAchr deficiency is associated with atherogenic changes in macrophage phenotype, including conditional increases in macrophage oxidative stress, oxidized LDL cellular uptake, and cholesterol mass accumulation, a clear feature of foam cell formation, and a hallmark of early atherosclerosis development. These findings suggest that means to increase the expression and activation of the α7nAchr could have therapeutic potential.

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