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Apocynin suppresses the progression of atherosclerosis in apoE-deficient mice by inactivation of macrophages

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ABSTRACT

Production of reactive oxygen species (ROS) and other proinflammatory substances by macrophages plays an important role in atherogenesis. Apocynin (4-hydroxy-3-methoxy-acetophenone), which is well known as a NADPH oxidase inhibitor, has anti-inflammatory effects including suppression of the generation of ROS. However, the suppressive effects of apocynin on the progression of atherosclerosis are not clearly understood. Thus, we investigated anti-atherosclerotic effects of apocynin using apolipoprotein E-deficient (apoE$^{-/-}$) mice *in vivo* and in mouse peritoneal macrophages *in vitro*. In atherosclerosis-prone apoE$^{-/-}$ mice, apocynin suppressed the progression of atherosclerosis, decreased 4-hydroxynonenal-positive area in atherosclerotic lesions, and mRNA expression of monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) in aorta. In mouse peritoneal macrophages, apocynin suppressed the Ox-LDL-induced ROS generation, mRNA expression of MCP-1, IL-6 and granulocyte/macrophage colony-stimulating factor, and cell proliferation. Moreover, immunohistochemical studies revealed that apocynin decreased the number of proliferating cell nuclear antigen-positive macrophages in atherosclerotic lesions of apoE$^{-/-}$ mice. These results suggested that apocynin suppressed the formation of atherosclerotic lesions, at least in part, by inactivation of macrophages. Therefore, apocynin may be a potential therapeutic material to prevent the progression of atherosclerosis.

1. Introduction

The progression of atherosclerosis and the stability of atherosclerotic plaques decide cardiovascular disease outcome. The development of atherosclerosis depends on a fragile balance between proinflammatory and oxidative stimuli on one phase, and anti-inflammatory and anti-oxidative defense mechanisms on the other phase [1,2]. Therefore, the control of reactive oxygen species (ROS) is one of the beneficial steps for the prevention of cardiovascular disease.

Apocynin (4-hydroxy-3-methoxy-acetophenone) is a constituent of the Himalayan herb *Picrorhiza kurroa* Royle (Scrophulariaceae) that is well known as a traditional Indian medicine (Ayurveda). It has been reported that apocynin has several anti-inflammatory effects such as the suppression of neutrophil oxidative burst, neutrophil-mediated oxidative tissue damage [3,4], monocyte adhesion to endothelial cells [5], chemotaxis of polymorphonuclear granulocyte [6], peroxynitrite production by macrophages [7] and cyclooxygenase-2 expression in human monocytes [8]. The underlying mechanism for these biological effects of apocynin is explained by the inhibition of NADPH oxidases, superoxide O_2^- generating enzymes [3,9].

NADPH oxidases play critical roles in the regulation of inflammatory processes by promoting oxidation of lipids and proteins, which ultimately leads to tissue damages and organ failure [10]. NADPH oxidases are expressed in all vascular cells, including endothelial cells (ECs), smooth muscle cells (SMCs), fibroblasts, and monocytes/macrophages [10,11]. Notably, NADPH oxidases also plays a key role in the development and progression of atherosclerotic lesions [12]. Interestingly, apocynin has been reported to have both NADPH oxidase-dependent and NADPH oxidase-independent anti-oxidative effects [13].

It is well known that atherosclerosis is a chronic inflammatory disease, and macrophages and macrophage-derived foam cells play pivotal roles in the progression of atherosclerosis by expressing inflammatory cytokines and chemokines [1]. Since apocynin has both anti-inflammatory and anti-oxidative effects, apocynin may suppress the progression of atherosclerosis via inactivation of macrophages. However, the suppressive effects of apocynin on

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the progression of atherosclerosis are not clearly understood. To address this issue, the present study was undertaken to determine the effect of apocynin on the expression of the inflammatory cytokines in macrophages and the progression of atherosclerosis in apolipoprotein E deficient (apoE$^{-/-}$) mice.

2. Materials and methods

2.1. Materials

Apocynin was purchased from Calbiochem (San Diego, CA). The antibodies against proliferating cell nuclear antigen (PCNA; Chromo Tek GmbH, Martinsried, Germany), 4-hydroxy-2-nonenal (4-HNE; NIKKEN SEIL Co., Ltd., Tokyo, Japan), and F4/80 (Abcam, Cambridge, UK) were from commercial sources. DAPI was from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of the best grade available from commercial sources.

2.2. Animals

ApoE$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). These mice were maintained on a C57BL/6 background. Mice were given free access to food and water in the Animal Resource Facility at Kumamoto University under specific pathogen-free conditions. All animal procedures were approved by the Animal Research Committee at Kumamoto University, and were conducted according to the Guide for the Care and Use of Laboratory Animals issued by the Institute of Laboratory Animal Resources. We used normal rodent chow for mice (CLEA, Tokyo, Japan) [14]. Twenty male mice of 6 weeks of age were treated orally with apocynin (100 mg/kg) (n = 11) or vehicle alone (water) as a control (n = 11). After 10 weeks of treatment, the mice were sacrificed and atherosclerotic lesions of aortic sinus were prepared for immunohistochemistry as described below. The whole aorta or 6-μm-thick frozen sections of the aortic sinus were obtained from ApoE$^{-/-}$ mice and were stained with Oil red O, as previously described [14]. Lesion size was measured on digital microphotographs of the aortic sinus by measuring the stained surface area using ImageJ software (NIH, Bethesda, MD). Plasma total cholesterol, triglyceride, and HDL cholesterol concentrations were measured at Skylight Biotech Inc., (Akita, Japan) [14].

2.3. Immunohistochemistry

The expression of PCNA, 4-HNE, and F4/80 was visualized on frozen aortic sinus sections by fluorescent immunohistochemistry. After equilibration in phosphate-buffered serine (PBS), 6-μm-thick frozen sections of the aortic sinus were blocked in 3% normal goat serum/2.5% Triton X-100 (NacalaiTesque, Kyoto, Japan)/PBS for 2 h at room temperature. Sections were then incubated overnight at 4 °C with the anti-PCNA, anti-4-HNE and anti-F4/80 antibodies diluted 1/100 in blocking solution. After six washes with PBS, the sections were incubated with Alexa Fluor 546 and 488 fluorescence-conjugated secondary antibodies (Molecular Probes, Eugene, OR) in blocking solution for 2 h at room temperature. The sections were then counterstained with DAPI solution. After rinsing with PBS, the sections were mounted with Fluoromount (Diagnostic BioSystems, Pleasanton, CA) and examined under a fluorescent microscope (Olympus, Tokyo, Japan) [14]. Immunohistochemical analyses were performed on six non-consecutive sections of the aortic sinus obtained from each of five animals.

2.4. Lipoprotein preparation

Human LDL (d = 1.019–1.063 g/ml) was isolated by ultracentrifugation of plasma samples obtained from normolipidemic subjects after an overnight fast [15]. LDL was dialyzed against 0.15 M NaCl and 1 mM EDTA (pH 7.4). Ox-LDL was prepared by incubating LDL with 5 μM $CuSO_4$ for 20 h at 37 °C, followed by the addition of 1 mM EDTA, and cooling [15]. The protein concentrations were determined using BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). The endotoxin level in the prepared Ox-LDL was <1 pg/μg protein, as measured using a Toxicolor System (Seikagaku Corp, Tokyo, Japan) [15].

2.5. Cell culture

Peritoneal macrophages were collected from anesthetized male C3H/He mice (25–30 g) by peritoneal lavage with 8 ml of PBS, centrifuged at 200×g for 5 min, resuspended in medium A (RPMI 1640; Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% fetal calf serum (Life Technologies Inc., Carlsbad, CA), 0.1 mg/ml streptomycin, and 100 U/ml penicillin), and then incubated in tissue culture plates for 90 min [15]. More than 98% of the adherent cells were considered to be macrophages based on four criteria, including (i) adherence to culture plates, (ii) morphologic features resembling mononuclear cells after Giemsa staining, (iii) the capacity to take up carbonparticles, and (iv) immunohistochemically positive for Cluster of Differentiation 68 (CD68), as previously described [16,17].

2.6. Tritiated thymidine incorporation and cell counting assays

Macrophage monolayers (2×10^6 cells/well) were cultured in 24-well tissue culture plates (15.5 mm diameter; Corning Glass Works, Corning, NY) in the presence of the indicated effectors for 6 days. For thymidine incorporation assays, the cells were incubated with 1 μCi/ml [^{3}H]thymidine for 18 h before terminating the experiments. Tritiated thymidine incorporation assays were performed as previously described [15]. For cell counting assays, cultured cells were lysed in 1% (w/v) Triton X-100, and naphthol blue–black-stained nuclei were counted in a hemocytometer, as previously described [15].

2.7. Measurement of intracellular ROS

The fluorescent probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (H_2DCF-DA) (C-2938; Molecular probes) was used for the assessment of intracellular ROS. Mouse macrophages (5×10^4 cells/well) were preincubated with indicated effectors for 1 h. After incubation with 20 μg/ml of Ox-LDL for 30 min, cells were incubated with 10 μM of H_2DCF-DA for 30 min, and analyzed in an HTS 7000 Bio Assay Fluorescent Plate Reader (Perkin Elmer-Cetus, Foster City, CA) using the HTSoft program as described previously [18]. ROS production was determined from an H_2O_2 standard curve (5–50 μM), and was expressed as a percentage of ROS incubated in normal conditions.

2.8. Real-time RT-PCR analysis

Total RNA was extracted with TRIzol (Life Technologies, Inc.) from cultured macrophages or atherosclerotic tissues of apoE$^{-/-}$ mice aorta. The first strand cDNA synthesis containing 1 μg of total RNA was primed with oligo dT. To quantify gene transcripts, the LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN) was used [14]. PCRs were performed using SYBR Green I master mix and specific primers for mouse monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), granulocyte/macrophage colony-stimulating factor (GM-CSF) and β-actin, which were designed as follows: MCP-1, forward primer, 5′-GGTCCCTGTCATGCTTCT-3′, and reverse primer, 5′-CATCTTGCTGGTGAATGAGT-3′; TNF-α, forward primer, 5′-AAATG

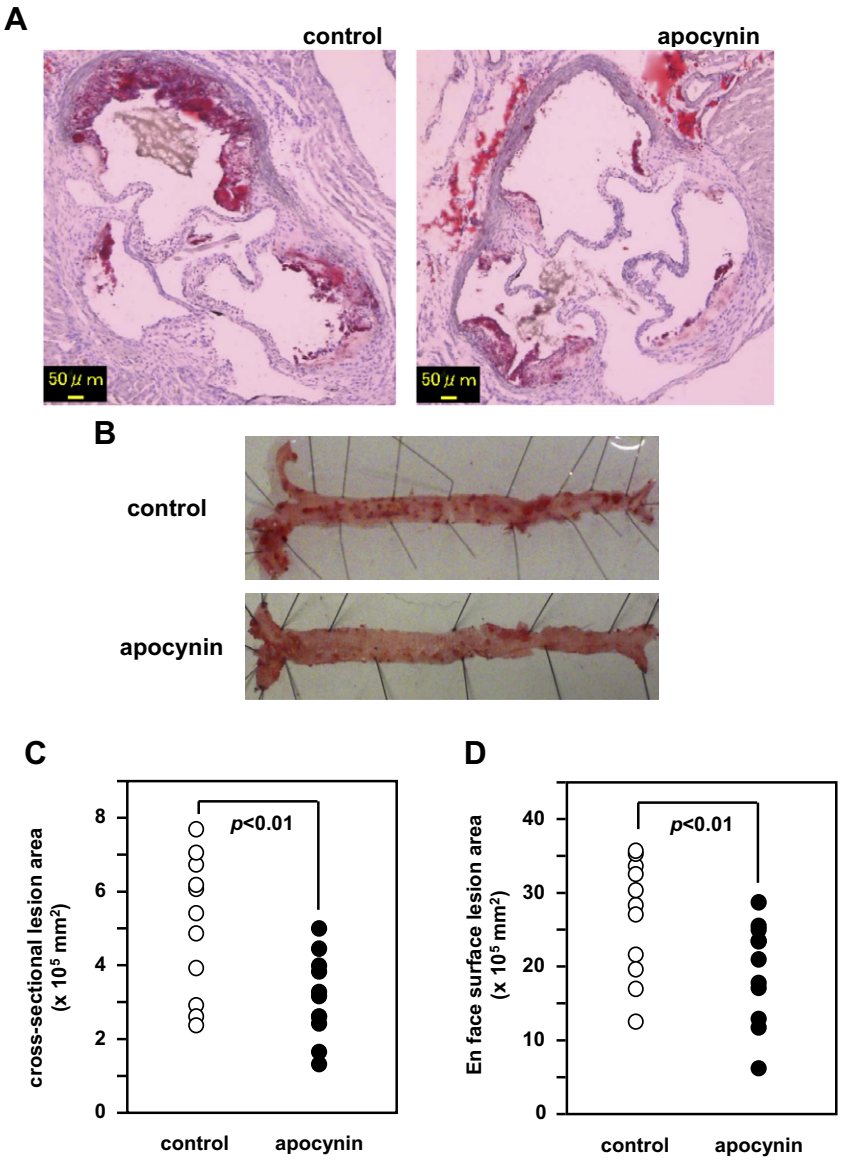

Fig. 1. Apocynin suppresses the progression of atherosclerosis in apoE$^{-/-}$ mice. ApoE$^{-/-}$ mice were treated without or with apocynin (100 mg/kg/day) for 10 weeks. (A and B), Representative photomicrographs of Oil red O-stained fatty streaks in the aortic sinuses (A) or the whole aortas (B) (original magnification,×5). (C and D) Quantitative analysis of atherosclerotic lesion size in cross-sections of the aortic sinus (C) or the whole aortas (D) in control and apocynin-treated apoE$^{-/-}$ mice (n = 11 mice/group).

GCCTCCCTCTCATCA-3′, and reverse primer, 5′-TTCTCCTGGTATGAGATAGC-3′; IL-6, forward primer, 5'-CCATCCAGTTGCCTTCTTGG-3′, and reverse primer, 5′-TGCAAGTGCATCATCGTTGT-3′; GM-CSF, forward primer, 5′-TGTGGTCTACAGCCTCTCAGCAC-3′, and reverse primer, 5′-CAAAGGGGATATCAGTCAGAAAGGT-3′; β-actin, forward primer, 5′-GTGGGCCGCTCTAGGCACCAA-3′, and reverse primer, 5′-CTCTTTGATGTCACGCACGATTTC-3′. The quantitative results for MCP-1, TNF-α, IL-6 and GM-CSF were normalized by the levels of β-actin mRNA [14,18]. To assess the specificity of the amplified PCR products, after the last cycle, a melting curve analysis was performed.

2.9. Statistical analysis

All values are means ± standard deviation of values from four independent experiments. The statistical significance of differences between groups was examined by one-factor analysis of variance followed by a post hoc Tukey test. Values of $p < 0.01$ were considered to indicate statistically significant differences.

3. Results

3.1. Apocynin ameliorates atherosclerotic lesion formation in apoE$^{-/-}$mice

To confirm whether apocynin could suppress the progression of atherosclerosis *in vivo*, we examined the effect of apocynin on atherosclerotic lesion formation in apoE$^{-/-}$ mice. Treatment with apocynin did not affect body weight and plasma levels of total cholesterol, triglyceride and HDL cholesterol in apoE$^{-/-}$ mice (data not shown). However, treatment with apocynin suppressed the formation of atherosclerotic lesions in the aortic sinus (Fig. 1A and C) and in the whole aorta (Fig. 1B and D), as determined by Oil red O staining.

As shown in Fig. 2A, 4-HNE-positive area in the atherosclerotic lesions was less in apocynin-treated mice than in control mice. Real-time RT-PCR assay revealed that mRNA expression of both MCP-1 and IL-6 was significantly decreased in apocynin-treated mice as compared with those in control mice (Fig. 2B and D).

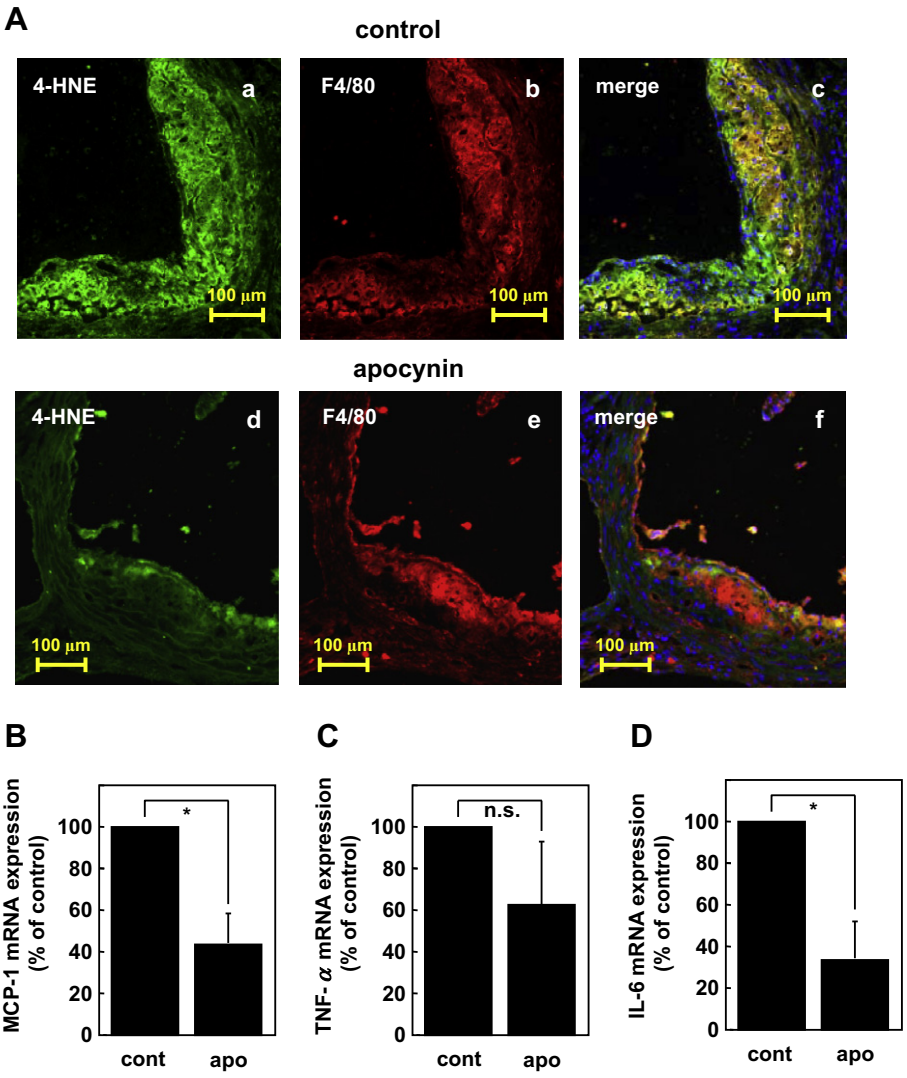

Fig. 2. Apocynin decreases 4-HNE-positive area in atherosclerotic lesion, and suppresses mRNA expression of inflammatory chemokine and cytokines. (A) Atherosclerotic lesions in the aortic sinuses from control (a, b, and c) or apocynin (apo)-treated (d, e, and f) apoE$^{-/-}$ mice were stained for 4-HNE (green; a, c, d and f), F4/80 (red; b, c, e and f), and DAPI (blue; c and f). (B, C and D) Aortas from control (a, b, and c) or apocynin (apo)-treated (d, e, and f) apoE$^{-/-}$ mice were collected. mRNA expression levels of MCP-1 (B), TNF-α (C), IL-6 (D) and β-actin were determined by real-time RT-PCR. Values are means ± SD of four separate experiments.*$P < 0.01$ vs. control mice.

Moreover, mRNA expression of TNF-α tended to be lower in apocynin-treated mice (Fig. 2C). These results suggested that apocynin suppressed the progression of atherosclerosis via inhibition of both ROS generation and production of pro-inflammatory mediators in apoE$^{-/-}$ mice.

3.2. Apocynin suppresses Ox-LDL-induced ROS generation, inflammatory cytokine expression and proliferation of macrophages

As shown in Fig. 3A, Ox-LDL (20 μg/ml) increased intracellular ROS generation in macrophages, which was suppressed by apocynin. Ox-LDL (20 μg/ml) significantly increased MCP-1, TNF-α and IL-6mRNA expression (Fig. 3B–D, respectively). Pre-treatment of apocynin (100 μM) significantly suppressed Ox-LDL-induced MCP-1 and IL-6 mRNA expression (Fig. 3B and D). Pre-treatment of apocynin tended to suppress Ox-LDL-induced TNF-α mRNA expression (Fig. 3C). We previously reported that Ox-LDL has a capacity to induce macrophage proliferation through the expression of GM-CSF [19,20]. Therefore, we next examined whether apocynin would suppress the Ox-LDL-induced GM-CSF expression and proliferation of macrophages. As shown in Fig. 3E, apocynin significantly suppressed Ox-LDL-induced [^{3}H]thymidine incorporation into macrophages in a dose-dependent manner. Cell counting assay also showed that Ox-LDL (20 μg/ml) increased cell number by 2.3 fold ($p < 0.01$, vs control), which was suppressed by the pretreatment of apocynin (100 μM) by 78% ($p < 0.01$, vs Ox-LDL alone). Moreover, apocynin(100 μM) significantly reduced Ox-LDL-induced GM-CSF mRNA expression (Fig. 3F). Therefore, apocynin might suppress Ox-LDL-induced macrophage proliferation by inhibiting GM-CSF expression.

To confirm whether macrophage proliferation was suppressed by apocynin treatment in atherosclerotic plaque, we next examined the effect of apocynin on proliferative cell number of macrophages in atherosclerotic lesions of apoE$^{-/-}$ mice. Fig. 4 showed that PCNA-positive and F4/80-positive cells were detected in the atherosclerotic lesions of apoE$^{-/-}$ mice and the number of PCNA and F4/80 double-positive cells in the atherosclerotic lesions was decreased in apocynin-treated mice as compared with that in control mice (Fig. 4A and B). These results suggested that apocynin suppressed the progression of atherosclerosis and macrophage function *in vivo* and *in vitro*.

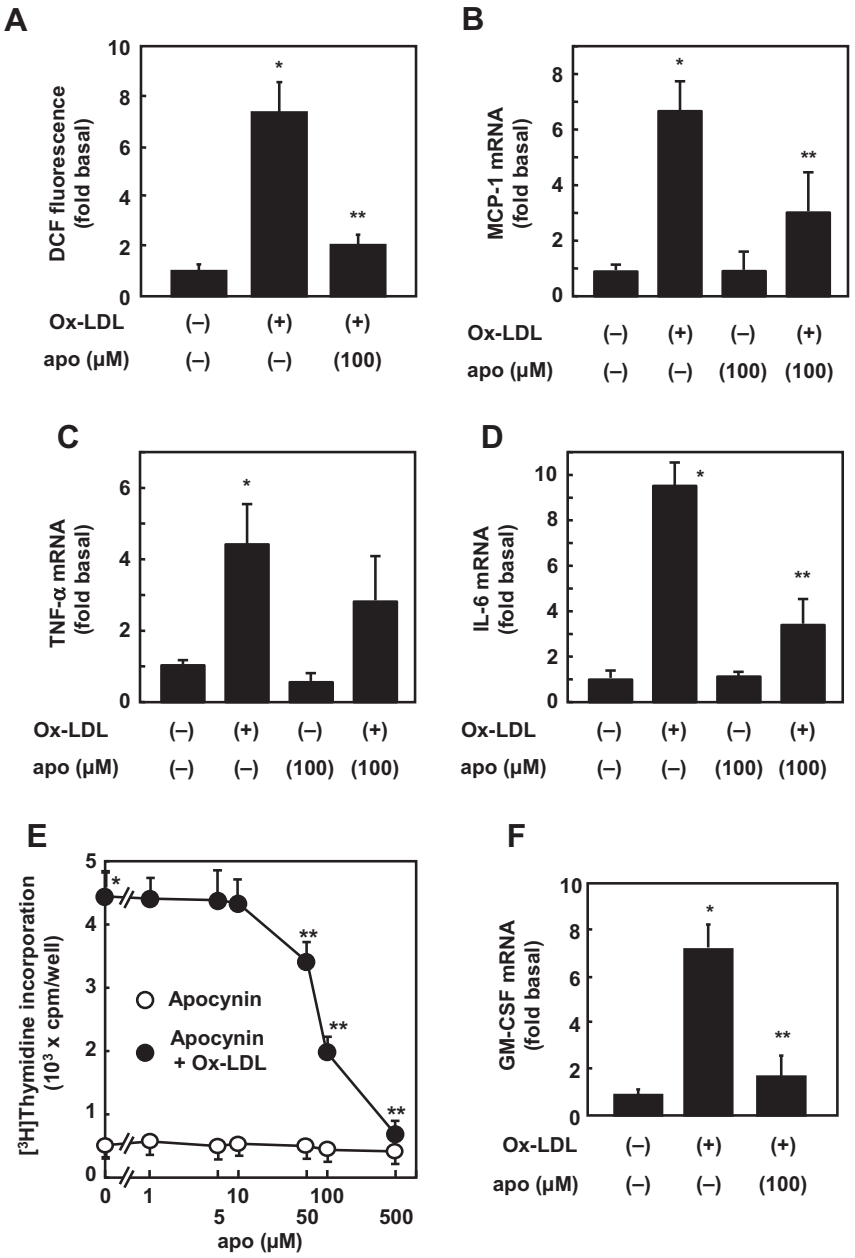

Fig. 3. Apocynin suppresses Ox-LDL-induced ROS generation, mRNA expression of inflammatory chemokine and cytokines, and proliferation of macrophages. Mouse peritoneal macrophages were incubated with 20 μg/ml Ox-LDL for 1 h (A) 4 h (B, C, D and E) or 6 days (D) in theabsence or presence of 100 μM apocynin (apo). (A), The intracellular ROS level was determined by the H_2DCF-DA method. Values are means ± SD of four separate experiments. (B, C, D and F) The mRNA expression levels of MCP-1 (B), TNF-α (C), IL-6 (D), GM-CSF (F) and β-actin were determined by real-time RT-PCR. Values are means ± SD of four separate experiments. (E) [^{3}H] thymidine incorporation assay was performed. Values are means ± SD of four separate experiments. $^*p < 0.01$ versus untreated cells. $^{**}p < 0.01$ versus Ox-LDL-treated cells.

4. Discussion

This study demonstrated that apocynin suppressed ROS generation and progression of atherosclerosis in apoE$^{-/-}$ mice. Our results also demonstrated that apocynin suppressed the Ox-LDL-induced ROS generation, cytokine and chemokine expression, and proliferation of macrophages. Vascular inflammation and cell proliferation in atherosclerotic lesions are closely linked [21], and excessive proliferation of vascular cells plays an important role in the pathobiology of vascular occlusive disease. On the other hand, enhanced oxidative stress seems to play a crucial role in this setting [2] by contributing to the inflammatory cascades in the vessel wall [22] and influencing cell cycle progression [23]. NADPH oxidase is one of the most prominent sources of vascular ROS, and NADPH oxidase-dependent ROS production is linked to atherogenesis. In the present study, we revealed that apocynin, which was known as an inhibitor of NADPH oxidase, suppressed the Ox-LDL-induced ROS generation, expression of atherogenic cytokines, and proliferation of macrophages. These results suggested that NADPH oxidase-mediated ROS generation might be involved in the formation of atheromatous plaques, and the suppression of NADPH oxidase might be one of the anti-atherogenic effects of apocynin.

Formation of ROS is one part of the unspecific defense systems against bacteria and other microbes. However, ROS may also affect cells in the host organism, in particular at sites of inflammation. Indeed, inflammation and oxidative stress seem to be closely linked [24]. Atherosclerosis is considered to be a chronic inflammatory

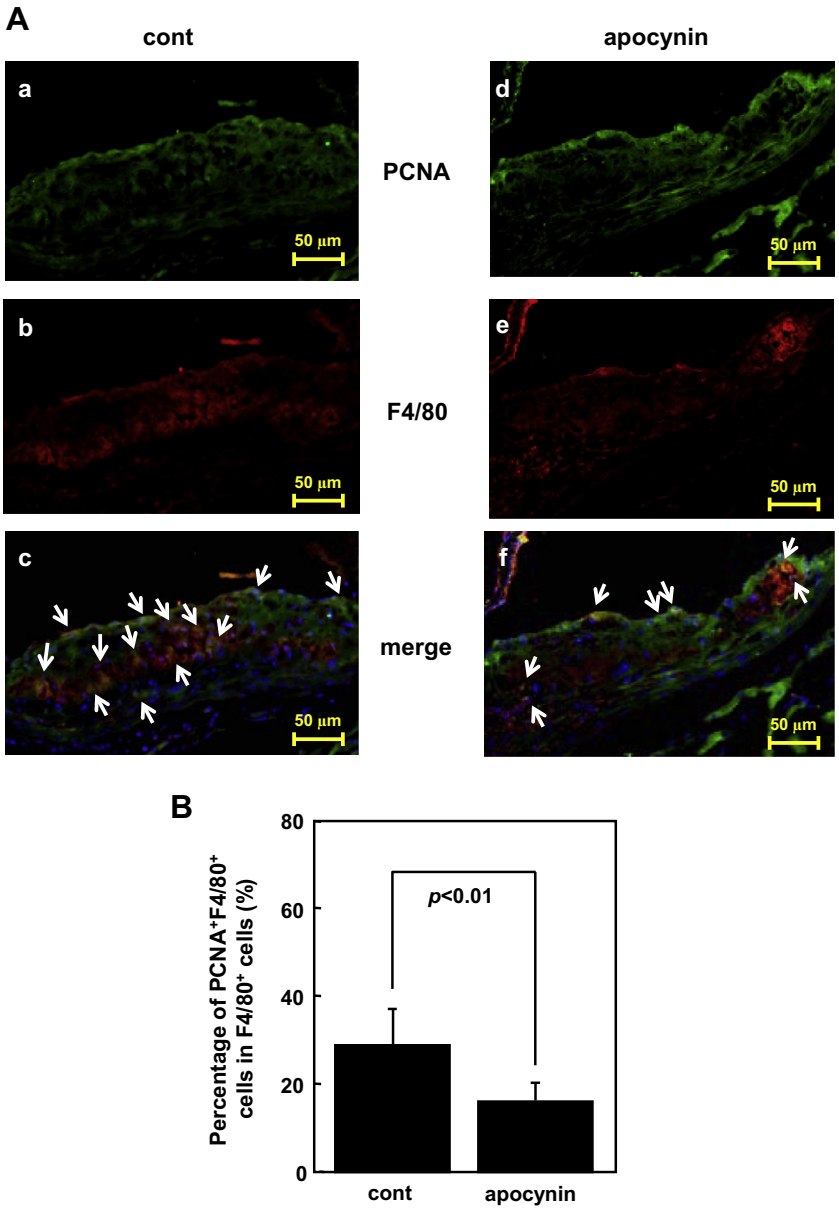

Fig. 4. Apocynin decreases the number of PCNA and F4/80 double-positive cells in atherosclerotic lesions. (A) Atherosclerotic lesions in the aortic sinuses from control (a, b, and c) or apocynin-treated (d, e, and f) apoE$^{-/-}$ mice were stained for PCNA (green; a, c, d, f), F4/80 (red; b, c, e and f) and DAPI (blue; c and f). Arrows indicate PCNA and F4/80 double-positive cells. (B) Percentage of PCNA and F4/80 double-positive cells of all F4/80-positive cells in the atherosclerotic lesions of control and apocynin-treated apoE$^{-/-}$ mice (12 sections; $n = 4$ mice/group).*$P < 0.01$ vs. control mice.

disease [1], and growing evidence indicates that chronic and acute overproduction of ROS under pathophysiological conditions is relevant for the development of cardiovascular diseases (CVD) [25]. In addition to NADPH oxidases, various sources for vascular ROS production have been identified. For example, ROS can be produced from xanthine oxidase, lipoxygenase, mitochondria, or the uncoupling nitric oxide synthase (NOS) [26]. Stimuli for enhanced vascular ROS formation are numerous, including pro-inflammatory mediators, such as Ox-LDL. Several lines of evidences indicate that Ox-LDL induces intracellular ROS generation through various pathways [26]. In the present study, we demonstrated that Ox-LDL increased intracellular ROS generation, and other reports revealed the presence of several pathways of the Ox-LDL-induced ROS generation in macrophages [27,28]. It has been reported that apocynin have anti-oxidative effects on not only NADPH oxidase-dependent but also NADPH oxidase-independent pathways [13]. Therefore, apocynin may be a beneficial compound for ROS-mediated formation of atherosclerotic lesions.

In vivo studies have reported that macrophage-derived foam cells proliferate in atherosclerotic lesions [29–31]. Moreover, we [15,32] and other researchers [33,34] have reported that Ox-LDL could also promote macrophage proliferation and survival *in vitro*. Based on the evidences mentioned above, it seems likely that Ox-LDL-induced macrophage proliferation is associated with enhanced progression of atherosclerosis. In terms of the growth signaling pathway, we previously reported that Ox-LDL-induced production of GM-CSF plays an important role in macrophage proliferation [19,35]. In the present study, we demonstrated that apocynin suppressed GM-CSF expression and cell proliferation in macrophages. These results suggested that the suppression of macrophage proliferation was a mechanism for the anti-atherogenic effects of apocynin.

In conclusion, we have demonstrated that apocynin suppressed ROS generation in atherosclerotic lesions and prevented the progression of atherosclerosis. We also showed that apocynin suppressed the Ox-LDL-induced expression of atherogenic chemokine and cytokines, and proliferation of macrophages. These results suggested that apocynin would be a potential therapeutic material to prevent the progression of atherosclerosis.

Acknowledgments

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References

[1] R. Ross, Atherosclerosis–an inflammatory disease, N. Engl. J. Med. 340 (1999) 115–126.
[2] V. Schachinger, A.M. Zeiher, Atherogenesis-recent insights into basicmechanisms and their clinical impact, Nephrol. Dial. Transplant. 17 (2002) 2055–2064.
[3] J.M. Simons, B.A. Hart, T.R. IpVaiChing, H. Van Dijk, R.P. Labadie, Metabolic activation of natural phenols into selective oxidative burstagonists by activated human neutrophils, Free Radic. Biol. Med. 8 (1990) 251–258.
[4] B.A. t Hart, J.M. Simons, S. Knaan-Shanzer, N.P. Bakker, R.P. Labadie, Antiarthritic activity of the newly developed neutrophil oxidativeburst antagonist apocynin, Free Radic. Biol. Med. 9 (1990) 127–131.
[5] C. Weber, W. Erl, A. Pietsch, M. Strobel, H.W. Ziegler-Heitbrock, P.C. Weber, Antioxidants inhibit monocyte adhesion by suppressing nuclearfactor-kappa B mobilization and induction of vascular cell adhesionmolecule-1 in endothelial cells stimulated to generate radicals, Arterioscler. Thromb. 14 (1994) 1665–1673.
[6] A.A. Muller, S.A. Reiter, K.G. Heider, H. Wagner, Plant-derivedacetophenones with antiasthmatic and anti-inflammatory properties: inhibitoryeffects on chemotaxis, right angle light scatter and actin polymerizationof polymorphonuclear granulocytes, Planta Med. 65 (1999) 590–594.
[7] R.B. Muijsers, E. van Den Worm, G. Folkerts, C.J. Beukelman, A.S. Koster, D.S.Postma, F.P. Nijkamp, Apocynin inhibits peroxynitriteformation by murine macrophages, Br. J. Pharmacol. 130 (2000) 932–936.
[8] S.S. Barbieri, V. Cavalca, S. Eligini, M. Brambilla, A. Caiani, E. Tremoli, S. Colli, Apocynin prevents cyclooxygenase 2 expression in human monocytes through NADPH oxidase and glutathione redox-dependent mechanisms, Free Radic. Biol. Med. 37 (2004) 156–165.
[9] J. Stolk, T.J. Hiltermann, J.H. Dijkman, A.J. Verhoeven, Characteristicsof the inhibition of NADPH oxidase activation in neutrophils by apocynin, amethoxy-substituted catechol, Am. J. Respir. Cell. Mol. Biol. 11 (1994) 95–102.
[10] K.K. Griendling, D. Sorescu, M. Ushio Fukai, NAD(P)H oxidase: role in cardiovascular biology and disease, Circ. Res. 86 (2000) 494–501.
[11] M. Bokoch, U.G. Knaus, NADPH oxidases: not just for leukocytes anymore!, Trends Biochem Sci. 28 (2003) 502–508.
[12] D. Sorescu, D. Weiss, B. Lassegue, R.E. Clempus, K. Szocs, G.P. Sorescu, L. Valppu, M.T. Quinn, J.D. Lambeth, J.D. Vega, W.R. Taylor, K.K. Griendling, Superoxide production and expression of nox family proteins in humanatherosclerosis, Circulation 105 (2002) 1429–1435.
[13] G.R. Drummond, S. Selemidis, K.K. Griendling, C.G. Sobey, Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets, Nat. Rev. Drug. Discov. 10 (2011) 453–471.
[14] T. Matsumura, H. Kinoshita, N. Ishii, K. Fukuda, H. Motoshima, T. Senokuchi, K. Taketa, S. Kawasaki, T. Nishimaki-Mogami, T. Kawada, T. Nishikawa, E. Araki, Telmisartan exerts anti-atherosclerotic effects by activating PPARγ in macrophages, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 1268–1275.
[15] T. Matsumura, M. Sakai, S. Kobori, T. Biwa, T. Takemura, H. Matsuda, H. Hakamata, S. Horiuchi, M. Shichiri, Two intracellular signaling pathways for activation of protein kinase C are involved in oxidized low-density lipoprotein-induced macrophage growth, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 3013–3020.
[16] J.A. Chodakewitz, T.S. Kupper, D.L. Coleman, Keratinocyte-derived granulocyte/macrophage colony-stimulating factor induces DNA synthesis by peritoneal macrophages, J. Immunol. 140 (1998) 832–836.
[17] R. Munker, J. Gasson, M. Ogawa, H.P. Koeffler, Recombinant human TNF induces production of granulocyte-monocyte colony-stimulating factor, Nature 323 (1986) 79–82.
[18] M. Yano, T. Matsumura, T. Senokuchi, N. Ishii, H. Motoshima, T. Taguchi, T. Matsuo, K. Sonoda, D. Kukidome, M. Sakai, T. Kawada, T. Nishikawa, E. Araki, Thiazolidinediones inhibit oxidized low density lipoprotein-induced macrophage proliferation, Atherosclerosis 191 (2007) 22–32.
[19] T. Matsumura, M. Sakai, K. Matsuda, N. Furukawa, K. Kaneko, M. Shichiri, Cis-acting DNA elements of mouse granulocyte/macrophage colony-stimulating factor gene responsive to oxidized low density lipoprotein, J. Biol. Chem. 274 (1999) 37665–37672.
[20] T. Senokuchi, T. Matsumura, M. Sakai, T. Matsuo, M. Yano, S. Kiritoshi, K. Sonoda, D. Kukidome, T. Nishikawa, E. Araki, Extracellular signal-regulated kinase and p38 mitogen-activated protein kinase mediate macrophage proliferation induced by oxidized low-density lipoprotein, Atherosclerosis 176 (2004) 233–245.
[21] V.J. Dzau, R.C. Braun-Dullaeus, D.G. Sedding, Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies, Nat. Med. 8 (2002) 1249–1256.
[22] J.A. Berliner, J.W. Heinecke, The role of oxidized lipoproteins in atherogenesis, Free Radic. Biol. Med. 20 (1996) 707–727.
[23] R.E. Shackelford, W.K. Kaufmann, R.S. Paules, Oxidative stress and cell cycle checkpoint function, Free Radic. Biol. Med. 28 (2000) 1387–1404.
[24] C.F. Mueller, K. Laude, J.S. McNally, D.G. Harrison, ATVB in focus: redox mechanismsin blood vessels, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 274–278.
[25] N.R. Madamanchi, A. Vendrov, M.S. Runge, Oxidative stress and vascular disease, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 29–38.
[26] J. Galle, T. Hansen-Hagge, C. Wanner, S. Seibold, Impact of oxidized low density lipoprotein on vascular cells, Atherosclerosis 185 (2006) 219–226.
[27] R. Nguyen-Khoa, Z. Massy, V. Witko-Sarsat, S. Canteloup, M. Kebebe, B. Lacour, T. Drüecke, B. Descamps-Latscha, Oxidized low-density lipoprotein induces macrophage respiratory burst via its protein moiety: a novel pathway in atherogenesis?, Biochem Biophys. Res. Commun. 263 (1999) 804–809.
[28] B. Fischer, A. von Knethen, B. Brüne, Dualism of oxidized lipoproteins in provoking and attenuating the oxidative burst in macrophages: role of peroxisome proliferator-activated receptor-γ, J. Immunol. 168 (2002) 2828–2834.
[29] M.E. Rosenfeld, R. Ross, Macrophage and smooth muscle cell proliferation in atherosclerotic lesions of WHHL and comparably hypercholesterolemic fat-fed rabbits, Arteriosclerosis 10 (1990) 680–687.
[30] L.G. Spagnoli, A. Orlandi, G. Santeusanio, Foam cells of the rabbit atherosclerotic plaque arrested in metaphase by colchicine show a macrophage phenotype, Atherosclerosis 88 (1991) 87–92.
[31] D. Gordon, M.A. Reidy, E.P. Benditt, S.M. Schwartz, Cell proliferation in human coronary arteries, Proc. Natl. Acad. Sci. USA 87 (1990) 4600–4604.
[32] M. Sakai, A. Miyazaki, H. Hakamata, T. Sasaki, S. Yui, M. Yamazaki, M. Shichiri, S. Horiuchi, Lysophosphatidylcholine plays an essential role in the mitogenic effect of oxidized low density lipoprotein on murine macrophages, J. Biol. Chem. 269 (1994) 31430–31435.
[33] J.S. Martens, N.E. Reiner, P. Herrera-Velit, U.P. Steinbrecher, Phosphatidylinositol 3-kinase is involved in the induction of macrophage growth by oxidized low density lipoprotein, J. Biol. Chem. 273 (1998) 4915–4920.
[34] J.A. Hamilton, D. Myers, W. Jessup, F. Cochrane, R. Byrne, G. Whitty, S. Moss, Oxidized LDL can induce macrophage survival, DNA synthesis, and enhanced proliferative response to CSF-1 and GM-CSF, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 98–105.
[35] T. Biwa, H. Hakamata, M. Sakai, A. Miyazaki, H. Suzuki, T. Kodama, M. Shichiri, S. Horiuchi, Induction of murine macrophage growth by oxidized low-density lipoprotein is mediated by granulocyte macrophage colony-stimulating factor, J. Biol. Chem. 273 (1998) 28305–28313.