Synthetic Retinoid Am80 Reduces Scavenger Receptor Expression and Atherosclerosis in Mice by Inhibiting IL-6

Norifumi Takeda, Ichiro Manabe, Takayuki Shindo, Hiroshi Iwata, Satoshi Iimuro, Hiroyuki Kagechika, Koichi Shudo, Ryozo Nagai

Background—Macrophage scavenger receptors facilitate the uptake of modified low-density lipoprotein (LDL), formation of foam cells, and development of atherosclerosis. Given that proinflammatory cytokines, including IL-6, can modulate the macrophage foaming process, the aim of the present study was to determine whether the synthetic retinoic acid receptor-α/β-specific agonist Am80, which is also an IL-6 inhibitor, can modulate macrophage lipid accumulation and foam cell formation.

Methods and Results—Am80 suppressed IL-6 production induced by 12-myristate 13-acetate (PMA) or angiotensin II in mouse Raw264 macrophages. It also suppressed expression of the 2 major scavenger receptors (scavenger receptor-A [SR-A] and CD36), in part by inhibiting IL-6, and inhibited macrophage foam cell formation. Systemic administration of Am80 led to reductions in the areas of atherosclerotic lesions and foam cell accumulation in the aortas of apolipoprotein E (apoE)-deficient mice and reduced serum concentrations of IL-6 and IL-1β without affecting body weights, serum lipid profiles or IL-10 levels.

Conclusions—Am80 suppresses scavenger receptor expression and macrophage foam cell formation in vitro and prevents atherogenesis in apoE-deficient mice in vivo. This suggests Am80 is a novel candidate agent that could be highly useful in the prevention and treatment of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2006;26:1177-1183.)

Key Words: macrophage ■ IL-6 ■ CD36 ■ scavenger receptor-A ■ retinoid

Macrophage foam cell formation is a hallmark of both early and late atherosclerotic lesions. During that process, macrophages take up modified low-density lipoprotein (LDL) via scavenger receptors in the vessel wall and release a variety of immune mediators, reactive oxygen species and proteases, thereby playing a pivotal role in atherogenesis. Two macrophage scavenger receptors, scavenger receptor-A (SR-A) and CD36, mediate the majority of modified LDL uptake and promote the development of atherogenesis. Indeed, mice lacking these 2 receptors do not accumulate esterified cholesterol derived from modified LDL. Thus, development of drugs that modulate scavenger receptor function could potentially provide a strong basis for a novel antiatherogenic therapy.

Proinflammatory cytokines are known to affect both the expression of scavenger receptors and the formation of macrophage foam cells. IL-6, for example, is expressed within atherosclerotic lesions in macrophage-rich areas and may stimulate inflammatory responses in macrophages, as well as proliferation of smooth muscle cells (SMCs) and pro-thrombotic activity. Recent studies have also shown that IL-6 plays a key role in angiotensin II (Ang II)-mediated CD36 expression and uptake of oxidized LDL in mouse peritoneal macrophages, and promotes atherogenesis. That mouse peritoneal macrophages obtained from IL-6−deficient mice do not show upregulation of CD36 expression in response to Ang II stimulation suggests inhibition of IL-6 is potentially promising therapeutic strategy for the treatment of atherosclerosis.

Am80 is a retinoic acid receptor (RAR) α/β-specific synthetic retinoid, and it neither binds to nor transactivates the retinoid X receptors (RXRs). Am80 is also known to inhibit the IL-6 signaling. Am80 suppresses IL-6 production in splenic mononuclear cells and reduces the severity and progression of inflammatory disease models, including 2,4-dinitrofluorobenzene–induced contact dermatitis, collagen-induced arthritis, and allergic encephalomyelitis. Recently, we reported that Am80 inhibits neointima formation in a mouse vascular injury model, suggesting it also modulates inflammatory and remodeling processes in the vessel wall. However, it is not yet known whether Am80 has the capacity to modulate macrophage function.

The aims of the present study were to determine whether Am80 can inhibit macrophage IL-6 production and foam cell formation and development of atherosclerosis in vivo.
formation in vitro, and whether treatment with Am80 can affect the development of atherosclerotic lesions in apolipoprotein E (apoE)-deficient mice in vivo. Our findings indicate that Am80’s ability to inhibit IL-6 expression enables it to suppress both macrophage foam cell formation and atherogenesis.

Materials and Methods

Plasmids

The IL-6 promoter reporter constructs pIL6-luc651, pIL6-luc651 ΔNF-kB, and pIL6-luc651 ΔC/EBPβ were a generous gift from Dr O. Eickelberg. The CD36 promoter reporter constructs pGL-CD36 (-273/luc), was a generous gift from Dr R.M. Evans. The RXRa expression vector CMX-hRXRa was a general gift from Dr R. Schule. The PPARγ expression vector pCAG-PPARγ was previously described.

For enhanced Materials and Methods used in this article, please see http://atvb.ahajournals.org.

Results

Am80 Inhibits IL-6 Expression

We first analyzed the effect of Am80 on production of IL-6 in macrophages. Raw264 cells were cultured with or without various concentrations of Am80 in the presence of 100 ng/mL PMA, a model agonist that induces scavenger receptor expression or 1 μmol/L Ang II. As shown in Figure 1A, PMA and Ang II induced significant IL-6 production and secretion in Raw264 cells, and this effect was dose-dependently inhibited by Am80. Likewise, Am80 dose-dependently inhibited PMA and Ang II–induced IL-6 mRNA expression (Figure 1B).

Am80 Inhibits the IL-6 Promoter Through C/EBPβ

Its inhibition of IL-6 mRNA expression suggested that Am80 may inhibit IL-6 gene expression at the level of transcription. To test that idea, we analyzed the effect of Am80 on IL-6 promoter activity. Raw264 cells were transfected with an IL-6 promoter-reporter construct (pIL6-luc651), after which the transfected cells were incubated with or without Am80 (10⁻⁷ mol/L) for 6 hour and then treated with PMA for 24 hour. As expected, PMA stimulated IL-6 promoter activity (Figure 1C). This effect was inhibited by Am80, further confirming that the retinoid suppresses IL-6 production at least in part by inhibiting IL-6 transcription.

The IL-6 promoter is known to be controlled by C/EBPβ (NF-IL6) and NF-κB. Because earlier studies have shown that ligand-bound RAR inhibits transactivation by C/EBPβ, we hypothesized that Am80 might suppress IL-6 transcription by inhibiting C/EBPβ. To test that idea, we transfected cells with mutant IL-6 promoter constructs in which either the C/EBPβ or NF-κB binding site was mutated. Mutation of the NF-κB binding site resulted in a significant (49%) reduction in IL-6 promoter activity, as compared with the wild-type construct under the basal culture conditions; mutation of the C/EBPβ binding site reduced activity to a slightly lesser degree (24%). PMA significantly increased the activity of both mutant promoter constructs. Am80, however, significantly reduced the reporter activity of the NF-κB site mutant (pIL6-luc651 ΔNF-κB) but had no effect on that of the C/EBPβ site mutant (pIL6-luc651 ΔC/EBPβ) (Figure 1C).

Am80 Reduces the Cholesterol Content and the Size and Number of Lipid Droplets in Mouse Peritoneal Macrophages

We next tested whether Am80’s ability to inhibit IL-6 production in macrophages might affect foam cell formation. When peritoneal macrophages were incubated with acety-
Am80 Suppresses Expression of SR-A and CD36 in Mouse Peritoneal and Raw264 Macrophages

Modified LDL promotes its own uptake into macrophages by upregulating the scavenger receptors SR-A and CD36. We therefore hypothesized that Am80 might affect expression of these receptors. To test that idea, we treated Raw264 macrophages with PMA, which is known to upregulate expression of both SR-A and CD36. As expected, PMA treatment increased mRNA expression of both of those genes in peritoneal macrophages, Raw264 cells (Abelson virus-transformed, murine macrophage-derived cell line) and THP-1 cells (human acute monocytic leukemia cell line) (Figure 3A). Am80 dose-dependently inhibited the PMA-induced expression of the 2 scavenger receptors in peritoneal macrophages and Raw264 cells and reduced expression of SR-A in THP-1 cells (Figure 3A). However, CD36 expression was somewhat upregulated by Am80 in THP-1 cells. Although the exact mechanism is unknown, the differential effect of Am80 on CD36 expression in macrophages (Raw264 and peritoneal macrophages) and monocytic THP-1 cells might reflect differences in species and in the differentiation state of the cells (see Discussion). Because the patterns of regulation of both SR-A and CD36 were similar in mouse peritoneal macrophages and Raw264 cells, we deemed Raw264 cells to be an appropriate model for use in the following experiments.

Like PMA, oxidized LDL upregulated SR-A and CD36 in Raw264 cells, and that upregulation was dose-dependently inhibited by Am80 (Figure 3B), which is consistent with the Am80-induced inhibition of cholesterol uptake by peritoneal macrophages seen in Figure 2. When we considered whether its inhibitory effect on IL-6 signaling might be involved in Am80’s inhibition of scavenger receptor expression, we found that addition of exogenous IL-6 partially restored expression of CD36 and SR-A mRNA, which was otherwise inhibited by Am80 (Figure 3C). Similarly, induction of the surface CD36 and SR-A proteins in Raw264 cells was inhibited by Am80 by flow cytometric analysis (Figure 3D), and this inhibition was partially restored by IL-6. These data demonstrate that Am80 acts to suppress expression of scavenger receptors at least in part via effects on IL-6 expression.

Am80 Inhibits the IL-6 Signaling

We then analyzed if Am80 might affect the IL-6-induced Cd36 and Sr-A expression. As expected, IL-6 upregulated expression of the scavenger receptor genes in Raw264 cells (Figure 4A). Am80 treatment resulted in decreases in the levels of the gene expression, suggesting Am80 might modulate the signaling mechanism that is elicited by IL-6 and leads to upregulation of CD-36 and SR-A.

To further analyze effects of Am80 on the signaling mechanism elicited by IL-6, we analyzed its effects on the CD36 promoter in Raw264 cells. As with the endogenous CD36 expression, CD36 promoter activity was augmented by IL-6 treatment (Figure 4B). This activation of the promoter was suppressed by Am80. These results suggest that Am80 affects both expression of IL-6 and the signaling elicited by IL-6.

Effect of Am80 on Atherosclerosis in ApoE-Deficient Mice

Given our observations that it inhibits IL-6 production and signaling elicited by IL-6, scavenger receptor expression and foam-cell formation in mouse macrophages, we hypothesized that Am80 might be able to modulate atherogenesis in vivo. To test that idea, 8-week-old apoE-deficient mice were fed a western diet for 2 months, during which they were orally administered Am80 (1.0 mg/kg body weight) or vehicle daily. There were no significant differences in the body weights or serum lipid profiles in the 2 groups (Table). Serum IL-6
levels were significantly reduced in the Am80-treated group, as expected. Levels of the proinflammatory cytokine IL-1β were also reduced in the Am80 group, but levels of the anti-inflammatory cytokine IL-10 were not. Fatty atherosclerotic lesions, measured as the percentage of the entire aorta affected or as the affected area of the aortic roots, were significantly smaller in the Am80-treated mice than in those receiving only vehicle (entire aorta: vehicle-treated, 7.3 ± 0.8%; Am80-treated, 0.5 ± 0.3%; P < 0.01; n = 8 in each group; aortic roots: vehicle-treated, 206 ± 12.352 μm²; Am80-treated, 149 ± 19.282 μm²; P < 0.01; n = 8 in each group) (Figure 5A to 5E). Immunohistochemical analysis showed that expression of IL-6 was decreased in plaques in the Am80-treated animals (Figure 5F). In addition, accumulation of extracellular matrix components around the aortic sinus and coronary artery was also reduced in Am80-treated mice (Figure 5G). Thus, Am80 does appear capable of inhibiting macrophage foam cell formation and atherogenesis in vivo.

Discussion

A hallmark of atherosclerotic lesions is the accumulation of macrophage foam cells, which play a central role in the development and progression of atherosclerosis. During that process, modified LDL is taken up primarily via 2 scavenger receptors, SR-A and CD36.2 Macrophages taken from mice lacking these 2 receptors fail to accumulate esterified cholesterol,3 suggesting these scavenger receptors represent potential therapeutic targets for inhibition of atherogenesis. In that regard, we have shown here that Am80 inhibits expression of scavenger receptors in mouse macrophages in vitro and substantially reduces atherosclerotic lesion formation in vivo.
Characteristics of ApoE-Deficient Mice Treated With Am80 on Western Diet for 2 Months

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Am80</th>
</tr>
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<tbody>
<tr>
<td>Initial body wt, g</td>
<td>22.43 ± 1.84</td>
<td>22.64 ± 1.64</td>
</tr>
<tr>
<td>Final body wt, g</td>
<td>27.25 ± 2.42</td>
<td>27.35 ± 2.17</td>
</tr>
<tr>
<td>Serum concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>1319 ± 127</td>
<td>1332 ± 143</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>165.3 ± 31.3</td>
<td>169.3 ± 27.1</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>62.8 ± 6.2</td>
<td>41.3 ± 6.3*</td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>132.6 ± 12.4</td>
<td>86.9 ± 15.3*</td>
</tr>
<tr>
<td>IL-10, pg/mL</td>
<td>36.3 ± 4.7</td>
<td>38.3 ± 5.2</td>
</tr>
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Results are expressed as the mean ± SD; n = 8, each group. *P < 0.05 vs vehicle-treated group.

Am80 has been used safely to treat acute promyelocytic leukemia,25 which would seem to make it an attractive candidate drug with which to treat and prevent atherosclerosis. Our findings demonstrate that Am80 inhibits IL-6 expression and the signaling elicited by IL-6 in macrophages. The central role played by IL-6 in cardiovascular disease is suggested by clinical studies showing that serum levels of the cytokine are increased in patients with unstable angina,2 that it is expressed in atherosclerotic lesions, and that it colocalizes with Ang II in the macrophage-rich shoulder region of plaques.4 IL-6 is thought to be a pivotal regulator of extracellular matrix deposition and reorganization.5 In addition, IL-6 has been shown to be involved in foam cell formation, and Keider et al reported that Ang II does not stimulate CD36 expression in peritoneal macrophages taken from IL-6-deficient mice, indicating that IL-6 is an important component of the signaling pathways that control CD36 expression.6 The results of the present study suggest that inhibition of IL-6 expression and signaling by Am80 reduces SR-A and CD36 expression in macrophages in vitro (Figure 3). Recent studies have shown that the JNK signaling is important for IL-6 expression in response to free cholesterol loading.26 However, Am80 did not alter JNK1/2 phosphorylation26 induced by the ACAT inhibitor (TMP-153) treatment27 in Raw264 cell (unpublished observations, Takeda and Manabe, 2005), suggesting that the JNK pathway is not involved in the inhibition of IL-6 expression by Am80.

Considering its various functions, Am80’s inhibition of IL-6 would be expected to affect both matrix degradation and foam cell formation in vivo. Systemically treating apoE-deficient mice with Am80 reduced accumulation of not only foam cells within atherosclerotic lesions but also extracellular matrix components (Figure 5). These findings suggest that inhibition of IL-6 production is a key mechanism by which Am80 suppresses plaque formation in apoE-deficient mice. Still, we and others have shown that Am80 also affects the functions of other cell types that play important roles in atherogenesis, including SMCs and T cells. For instance, Am80 suppresses expression of PDGF-A in SMCs by inhibiting KLF5,12 and it induces IL-10 secretion in T-cells.28 In addition, atRA has been shown to promote fibrinolysis and to inhibit thrombosis and platelet aggregation.29 Given that atherogenesis is an integral of a variety of cellular activities involving multiple cell types and various growth factors and cytokines, it is very likely that it is Am80’s cumulative effects on all affected cell types that lead to reduced plaque formation in apoE-deficient mice.

Figure 5. Am80 reduces plaque formation in apoE-deficient mice. Representative photographs showing the appearance of the aortic arch (A) and en face atherosclerotic lesions over the entire aorta stained with Sudan IV (B). C, Percentage of aortic area affected by en face atherosclerotic lesions (n = 8). Representative photomicrographs of the aortic sinus stained with Oil Red O (D), IL-6 antibody (F), and Masson trichrome stains (G). E, Area affected by atherosclerotic lesions in the aortic sinus. The scale bar indicates 100 μm (F). Data are means ± SD. *P < 0.05, **P < 0.01 vs vehicle-treated group.
treated animals. As mentioned above, Am80 affects the functions of a variety of cell-types, presumably via both IL-6-independent and -dependent mechanisms. These differences in effects of Am80 and null mutation of IL-6 are likely to have led to differential effects on plaque formation in apoE−/− mice.

The present findings are also at variance with earlier studies showing that RA induces CD36 in human monocyctic THP-1 cells,12–14 and IL-6 inhibits SR-A expression in THP-1 cells and human peripheral monocytes.15 This discrepancy may reflect differences between the models, the species, and the differentiation state of the cells. THP-1 cells are a monocytic cell line in which expression of SR-A and CD36 accompanies differentiation into macrophages. Raw264 cells and peritoneal macrophages, by contrast, are mouse macrophages and express CD36 even under basal culture conditions. Previous studies have shown that atRA promotes macrophage differentiation of THP-1 cells.16 We found that Am80 also promotes macrophage differentiation of THP-1 cells (unpublished observations, Takeda and Manabe, 2005). It is therefore plausible that the upregulation of CD36 seen in Figure 3A reflects differentiation, though the exact mechanisms underlying the differential effects of Am80 on CD36 remain unknown.

The results of our reporter assays suggest that Am80 suppresses IL-6 production at least in part at the level of transcription by inhibiting C/EBPβ-dependent transactivation of the IL-6 promoter. In Raw264 cells, PMA induced both IL-6 and C/EBPβ (Figure 1 and Figure I), whereas Am80 inhibited the PMA-induced IL-6 expression but not the C/EBPβ expression. This suggests that Am80 in some way interferes with the function of C/EBPβ. Consistent with this idea, C/EBPβ-dependent gene transcription is similarly inhibited by RA in adipocytes, although C/EBPβ expression is not.17

Tontonoz et al recently demonstrated that CD36 gene is controlled by PPARγ via the PPARγ/RXR-responsive element (PPRE) within −2741 to −263 bp region of the CD36 promoter.18 However, we found that the PPRE was dispensable for inhibition of the promoter activity by Am80. Moreover, Am80 did not affect the activity of PPRE-dependent minimal promoter18 (Takeda and Manabe, unpublished observations, 2005). These results suggest that Am80 inhibits the CD36 transcription via mechanisms independent of PPARγ. It is noteworthy to mention that the CD36 promoter has neither RARE nor the C/EBP binding motif. It would be important to determine the molecular mechanisms by which Am80 inhibits CD36 transcription in future studies for better understanding the role played by RAR in the control of macrophage function. Of particular importance will be RAR’s interactions with other transcription factors.

In conclusion, our findings support the notion that modulation of the function of inflammatory and vascular cells using synthetic retinoids is a promising strategy for the treatment and prevention of vascular diseases, including atherosclerosis.

Acknowledgments

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References


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Enhanced Materials and Methods

Plasmids
The IL-6 promoter reporter constructs pIL-6-luc651, pIL6-luc651 ΔNF-κB, and pIL6-luc651 ΔC/EBPβ were a generous gift from Dr O. Eickelberg. The CD36 promoter reporter constructs pGL-CD36(-273/luc), was a generous gift from Dr RM. Evans. The RXRα expression vector CMX-hRXRα was a general gift from Dr R. Schule. The PPARγ expression vector pCAG-PPARγ was previously described.

Preparation of Lipoproteins
Low density lipoprotein (LDL, density = 1.063-1.210 g/mL) was isolated from the plasma of healthy, fasting volunteers by sequential density ultracentrifugation, as described previously. For oxidative modification, LDL was incubated with CuSO₄ at 37 ºC. LDL was acetylated by repetitive additions of acetic anhydride.

Cell Culture
The Raw264 mouse macrophage cell line was purchased from RIKEN (Japan) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) FBS. THP-1 human monocytic leukemia cell line was purchased from ATCC (Manassas) and cultured in RPMI-1640 containing 10% (v/v) FBS and differentiated to THP-1 macrophages by the treatment with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 h. Mouse peritoneal macrophages were obtained from the peritoneal cavity of male C57Bl/6 mice (8-week-old) 4 days after injection of 4% thioglycolate (Sigma). The cells were washed, resuspended in RPMI-1640 supplemented with 10% (v/v) FBS, and plated. Non-adherent cells were removed by washing 1 hour after plating.

Cholesterol Determination
Cellular lipids were extracted using hexane/isopropanol, evaporated and dissolved in isopropanol. The cholesterol mass was quantified by enzymatic fluorometric microassay using to the method of Heider and Boyett with minor modifications. The amount of esterified cholesterol was calculated by subtracting the free cholesterol from the total.
cholesterol. The amount of cellular protein was quantified using BCA Protein Assay Reagent (Pierce) after dissolving the cells in 0.1 N NaOH.

**Oil Red O Staining**
Mouse peritoneal macrophages were cultured in 4-chamber plates at 1.0x10^6 cells/chamber and treated with modified LDL and Am80. The cells were then washed twice with PBS, fixed with 4% (w/v) paraformaldehyde in PBS, stained with Oil Red O in 60% (v/v) isopropanol, and counterstained with hematoxylin.

**RNA extraction and Quantification**
Total RNA was purified from cells using an RNeasy Mini kit (Qiagen). The methods for reverse transcription of RNA has been described. For quantitation of the transcripts, real-time PCR was carried out in a LightCycler (Roche) using a QuantiTect SYBR green PCR kit (Qiagen). The expression level of each gene was normalized to that of 18s rRNA, which served as an endogenous internal control. The sequences of the PCR primers for C/EBPβ have been published previously. The sequences of the PCR primers were: mouse IL-6, 5'- agttgccttcttgactga -3' and 5'- tccagatcctgagacgac-3'; mouse SR-A, 5'-ctggacaaactggtccacct-3' and 5'-tccccttttctctcctt-3'; mouse CD36, 5'-gagcaactggagtggtctgttg3' and 5'-gcagaatcaagggagacac-3'; human SR-A(I/II), 5'-cccgtgtttgcagttctca-3' and 5'-ccatgttgctcatgtgttcc-3'; human CD36, 5'-agatgcagcctcatttccac-3' and 5'-gccttgaggaagaaca-3'.

**Measurement of Biochemical Parameters**
IL-6 released from cells into the medium, and serum concentrations of IL-6, IL-1β and IL-10 were measured using a BioSource mouse ELISA kit following the manufacturer’s protocol. Other serum biochemical parameters were determined using commercially available kits (Wako Pure Chemicals).

**Transfection of Cells and Measurement of Luciferase Activity**
Raw264 cells were transfected with indicated reporter and expression vector plasmids using Lipofectamine 2000 (Invitrogen). To correct for variation in transfection
efficiency, we co-transfected 100 ng of pCMV-βgal in all experiments. Luciferase activity was assayed (Promega) luminometrically, and β-galactosidase activity was evaluated as described previously. The ratio of the luciferase activity to the β-galactosidase activity in each sample served as a measure of the normalized luciferase activity.

Flow Cytometry
To detect CD36 expression, cells were harvested, washed with PBS/3%BSA, and then incubated with phycoerythrin (PE)-conjugated anti-CD36 (Santa Cruz) antibody. To detect SR-A expression, the cells were incubated with anti-SR-A (clone 2F8, Cell Sciences) antibody, followed by a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Vector). Labeled cells were analyzed on a FACSCalibur flow cytometer using the CellQuest software program (BD Biosciences).

Animals
C57Bl/6 and ApoE-deficient mice (hybrids of the C57Bl/6 and 129Sv strains) were obtained from the Jackson Laboratories. All experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments.

Atherosclerosis in ApoE-Deficient Mice
Eight-week-old ApoE-deficient mice were fed an atherogenic diet containing 20% (wt/wt) fat with 0.15% (wt/wt) cholesterol. After the mice were killed, the extent of fatty streak formation was evaluated by measuring (1) the area of the en face surface lesion in the entire aorta and (2) the cross-sectional lesion area at the aortic root. Briefly, for evaluation of the entire aorta, the aorta was dissected from the aortic sinus to the iliac bifurcation, and lipid-rich atheroma was visualized by staining with Sudan IV. To assess cross-sectional lesions of the aortic sinuses, 5 serial sections at intervals of 50 µm were prepared and analyzed by Oil Red O staining and immunohistochemistry. Air-dried cryostat sections were fixed in acetone and stained with anti-mouse IL-6 antibody (BD PharMingen). After incubation with biotinylated secondary antibody (DakoCytomation), the sections were incubated with horseradish peroxidase-labeled
streptavidin solution (DakoCytomation) and visualized using 3-3’ diaminobenzidine (DAB). The sections were then counterstained with Mayer’s hematoxylin.

**Statistical Analyses**

All values in the text and figures represent means ± S.D. Statistical analysis of the data was carried out using Student’s unpaired $t$ test or 1-way ANOVA followed by Bonferroni/Dunn test for multiple comparisons. Values of $P<0.05$ were considered significant.
References


Supplemental Figure I.

Am80 does not affect PMA-induced C/EBPβ expression

Raw264 cells were pretreated with or without Am80, after which expression of C/EBPβ were assessed by real-time PCR at the indicated times after PMA treatment. Data are means ± S.D. of three independent experiments. #P < 0.05, ##P < 0.01 vs. PMA untreated control.