Nifedipine Induces Peroxisome Proliferator-Activated Receptor-γ Activation in Macrophages and Suppresses the Progression of Atherosclerosis in Apolipoprotein E-Deficient Mice


Objective—Nifedipine, an L-type calcium channel blocker, protects against the progression of atherosclerosis. We investigated the molecular basis of the antiatherosclerotic effect of nifedipine in macrophages and apolipoprotein E-deficient mice.

Methods and Results—In macrophages, nifedipine increased peroxisome proliferator-activated receptor-γ (PPARγ) activity without increasing PPARγ-binding activity. Amlodipine, another L-type calcium channel blocker, and 1,2-bis-(o-aminophenoxy)-ethane-N,N',N'-tetraacetic acid tetraacetoxy-methyl ester (BAPTA-AM), a calcium chelator, decreased PPARγ activity, suggesting that nifedipine does not activate PPARγ via calcium channel blocker activity. Inactivation of extracellular signal-regulated kinase 1/2 suppressed PPARγ2-Ser112 phosphorylation and induced PPARγ activity. Nifedipine suppressed extracellular signal-regulated kinase 1/2 activation and PPARγ2-Ser112 phosphorylation, and mutating PPARγ2-Ser112 to Ala abrogated nifedipine-mediated PPARγ activation. These results suggested that nifedipine inhibited extracellular signal-regulated kinase 1/2 activity and PPARγ2-Ser112 phosphorylation, leading to PPARγ activation. Nifedipine inhibited lipopolysaccharide-induced monocyte chemoattractant protein-1 expression and induced ATP-binding cassette transporter A1 mRNA expression, and these effects were abrogated by small interfering RNA for PPARγ. Furthermore, in apolipoprotein E-deficient mice, nifedipine treatment decreased atherosclerotic lesion size, phosphorylation of PPARγ2-Ser112 and extracellular signal-regulated kinase 1/2, and monocyte chemoattractant protein-1 mRNA expression and increased ATP-binding cassette transporter A1 expression in the aorta.

Conclusion—Nifedipine unlike amlodipine inhibits PPARγ-Ser phosphorylation and activates PPARγ to suppress monocyte chemoattractant protein-1 expression and induce ATP-binding cassette transporter A1 expression in macrophages. These effects may induce antiatherogenic effects in hypertensive patients. (Arterioscler Thromb Vasc Biol. 2010;30:1598-1605.)

Key Words: atherosclerosis ■ macrophages ■ hypertension ■ peroxisome proliferator-activated receptor ■ vascular biology

Hypertension is a major risk factor for atherosclerosis.1,2 However, the detailed mechanisms that link elevated blood pressure to progression of atherosclerosis remain unclear. Hypertension may promote the expression of cell adhesion molecules in endothelial cells3 and production of cytokines in the vessel wall, suggesting that hypertensive stress stimulates a proinflammatory response and contributes to the progression of atherogenesis. On the other hand, low-density lipoprotein (LDL) derived from hypertensive patients is more susceptible to lipid peroxidation mediated by angiotensin II and to its cellular uptake by macrophages than that obtained from normotensive subjects.5 Therefore, monocyte-derived macrophages and macrophage-derived foam cells may accelerate atherosclerosis in hypertensive patients. Massive clustering of macrophage-derived foam cells in the subendothelial spaces of arterial walls is a characteristic feature of early atherosclerotic lesions.6 Monocytes subsequently adhere to the endothelial cells, cross the endothelial layer to enter the subendothelial space, differentiate into macrophages, and eventually become foam cells. Macrophage-derived foam cells express increased PPARγ activity,7 suggesting that the expression of PPARγ may be important in the progression of atherosclerosis.

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Received on: December 21, 2009; final version accepted on: May 7, 2010.
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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.109.202309

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phage-derived foam cells are characterized by the accumulation of cholesteryl esters resulting from the uptake of modified lipoproteins through macrophage scavenger receptors. Foam cells produce bioactive molecules, such as cytokines and growth factors, and contribute to the development and progression of atherosclerosis.

Calcium channel blockers (CCBs) are relevant treatments for patients with hypertension. Beyond causing vasodilatation by inhibiting calcium channels, long-acting L-type CCBs produce clinical benefits in patients with coronary artery disease that might be independent of blood pressure changes. In fact, in vivo and in vitro studies demonstrated that L-type CCBs induce NO production in coronary microvessels and inhibit smooth muscle cell (SMC) migration and proliferation. Therefore, L-type CCBs may be antiatherogenic without their antihypertensive effects.

Peroxisome proliferator-activated receptor-γ (PPARγ) is a low-affinity dietary lipid receptor (reviewed by Rosen and Spiegelman in Reference 11). After binding to a ligand (e.g., thiazolidinediones, arachidonate, 15-hydroxyeicosatetraenoic acid, or others), it forms a heterodimer with the 𝜀-retinoic acid retinoid X receptor, binds to a PPAR response element, and activates gene transcription. PPARγ regulates gene expression in cell types that exist in atherosclerotic lesions, such as macrophages, endothelial cells, and vascular SMCs. PPARγ has many antiatherogenic effects in these cells. In addition, PPARγ agonists inhibit the development of atherosclerosis in LDL receptor-deficient mice and in apolipoprotein E-deficient (apoE−/−) mice. Thus, PPARγ activation is beneficial for suppressing atherosclerosis.

Nifedipine, an L-type CCB, reduced the atherosclerotic plaque area in cholesterol-fed rabbits and suppressed the progression of atherosclerosis in hypertensive patients. However, the mechanism(s) of nifedipine-induced antiatherosclerotic activity are not fully understood. We wished to clarify whether the antiatherogenic effect of nifedipine is mediated by PPARγ activation. We revealed that nifedipine activates PPARγ through inactivating extracellular signal-regulated kinase 1/2 (ERK1/2) and subsequently decreasing serine phosphorylation of PPARγ in macrophages. Moreover, nifedipine suppressed lipopolysaccharide (LPS)-induced monocyte chemoattractant protein-1 (MCP-1) expression and induced ATP-binding cassette transporter A1 (ABCA1) expression through PPARγ activation. Furthermore, nifedipine suppressed the progression of atherosclerosis in apoE−/− mice.

Methods

Materials

PD98059 and ciglitazone were purchased from Calbiochem. Nifedipine, fenofibrate, bezafibrate, and pioglitazone were kindly given from Bayer Yakuhin, Ltd., Kaken Pharmaceutical Co., Ltd, Kissei Pharmaceutical Co., Ltd., and Takeda Pharmaceutical, respectively. Amlodipine, 1,2-bis-(o-aminophenoxo)-ethane-N,N,N′,N′ ′-tetraacetic acid tetraacetoxy-methyl ester (BAPTA-AM) and U0126 were purchased from BIOMOL International, L.P. LPS (Escherichia coli O111:B4) was purchased from Sigma. Rabbit polyclonal anti-ERK1/2, anti-PPARγ, anti-PPARγ2 and anti-phospho PPARγ2 antibodies, and goat polyclonal anti-β-actin antibody were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-phospho ERK1/2 antibody was purchased from Cell Signaling Technology. Rabbit polyclonal anti-ABCA1 antibody was purchased from Novus Biologicals, Inc. All other chemicals were of the best grade available from commercial sources.

Cell Culture

Peritoneal macrophages were collected from anesthetized male C3H/He mice (25 to 30 g body weight) by peritoneal lavage and were resuspended and incubated in medium A (RPMI medium 1640 (Nissui Seiyaku) supplemented with 10% FCS (Invitrogen Japan K.K.)/0.1 mg/mL streptomycin/100 U/mL penicillin). More than 98% of the adherent cells were macrophages based on 4 criteria, as described previously. RAW264.7 cells were cultured in medium A in appropriate tissue culture plates as previously described. THP-1 cells were cultured in medium A in 35-mm dishes and treated with 200 nmol/L of phorbol myristate acetate to induce their differentiation into macrophages. After 8 hours of incubation, the cells were washed twice with PBS and incubated in medium A until the initiation of experiments.

Animals, full-length PPARγ luciferase assay system, PPARγ GAL4 chimera assay system (measurement of PPARγ ligand-binding activity), luciferase assays, transcriptional activity of PPARγ, transfection of small interfering RNA (siRNA) and plasmids, adenoviral vectors, cell transfection, Western blot analysis, reverse transcription and quantitative real-time polymerase chain reaction, enzyme-linked immunosorbent assay for MCP-1, lipoprotein preparation, determination of cellular cholesterol content, and statistical analysis are described in the supplemental information, available online at http://atvb.ahajournals.org.

Results

Full-Length PPARγ Luciferase Assay System Is Able to Measure PPARγ Activity

RAW264.7 cells lack PPARγ expression. To confirm whether RAW264.7 cells are suitable for our experiments, we first investigated the expression of PPARγ and the effect of the PPARγ agonists, ciglitazone and pioglitazone, on PPARγ activity using an (acetyl-coenzyme A oxidase)3-luciferase [(AOX)3-luc] plasmid containing a PPAR response element of AOX. Expression of PPARγ1 and PPARγ2 proteins was confirmed by Western blot analysis in RAW264.7 cells, as well as in mouse peritoneal macrophages (Figure 1A). However, ciglitazone and pioglitazone increased the luciferase activity in mouse peritoneal macrophages (Figure 1B) but not in RAW264.7 cells (Figure 1C). Therefore, ligand-dependent PPARγ activation was not detectable in RAW264.7 cells transfected with (AOX)3-luc plasmids alone.

To construct a full-length PPARγ luciferase assay system, RAW264.7 cells were transfected with plasmid human cytomegalovirus X (pCMX)-PPARγ2 and (AOX)3-luc. We then investigated luciferase activity using the PPARα agonists, bezafibrate and fenofibrate, and the PPARγ agonists, ciglitazone and pioglitazone. Basal luciferase activity was 7-fold higher in cells overexpressing PPARγ2 (Figure 1C). Moreover, 1 μmol/L of ciglitazone and pioglitazone increased this luciferase activity, whereas 5 μmol/L of fenofibrate and 5 μmol/L of bezafibrate did not (Figure 1C). These results suggest that our full-length PPARγ luciferase assay system could selectively measure PPARγ activity.

Nifedipine Activates PPARγ in Macrophages

Nifedipine dose dependently (<1 nmol/L) increased luciferase activity in this same assay system (Figure 2A), with less
activity at higher (10 nmol/L) levels. This activity was additive with pioglitazone or ciglitazone (Figure 2B), but nifedipine did not affect PPARγ ligand-binding activity (Figure 2C). These results suggest that nifedipine is not a direct ligand for PPARγ, or it did not increase intracellular PPARγ ligands. Similar concentrations (1 nmol/L) of nifedipine increased PPARγ activity by 3.3-fold in mouse peritoneal macrophages (*p < 0.01 versus control) and 3.8-fold in THP-1 macrophages (p < 0.01 versus control). Interestingly, amlodipine, another L-type CCB, decreased PPARγ activity (Figure 2D). BATPA-AM, an intracellular calcium chelator, also decreased PPARγ activity (Figure 2E).

Nifedipine Suppresses PPARγ Phosphorylation to Activate It

PPARγ activity is suppressed by mitogen-activated protein kinase-dependent phosphorylation.31,32 Mutating Ser112 to Ala within the putative ERK1/2 site in mouse PPARγ2 (pCMX-PPARγ2-S112A) abrogated ERK1/2-mediated inhibition of PPARγ activation.32 We hypothesized that nifedipine suppressed ERK1/2 activation, thereby decreasing PPARγ phosphorylation and increasing PPARγ activation. Although nifedipine significantly increased luciferase activity in cells overexpressing wild-type PPARγ2, nifedipine did not further enhance luciferase activity in cells overexpressing PPARγ2-S112A (Figure 3A). Moreover, nifedipine dose dependently suppressed phosphorylation of PPARγ2 in mouse peritoneal macrophages (Figure 3B) and in RAW264.7 cells overexpressing PPARγ2 (Figure 3C).

Inactivation of ERK1/2 Is Involved in Nifedipine-Induced PPARγ Activation

The phosphorylation of Ser112 of PPARγ2 is mediated by ERK1/2.31,32 Consistent with this finding, the MEK1/2-specific inhibitor, PD98059, and dominant negative-ERK suppressed phosphorylation of PPARγ2 in mouse peritoneal macrophages (Figure 4A). PD98059 also suppressed phosphorylation of PPARγ2 in RAW264.7 cells expressing PPARγ2 (Figure 4B). Moreover, PD98059 and the ERK1/2 inhibitor, U0126, increased PPARγ activity (Figure 4C) in RAW264.7 cells. Transfection of dominant negative-ERK...
also increased PPARγ activity (Figure 4D) in RAW264.7 cells, suggesting that ERK1/2 inhibition decreases PPARγ phosphorylation, leading to its activation. Nifedipine dose dependently blocked ERK1/2 phosphorylation in mouse peritoneal macrophages (Figure 4E) and RAW264.7 cells (Figure 4F). Moreover, overexpression of constitutively active MEKK enhanced phosphorylation of ERK1/2 in RAW264.7 cells, and nifedipine-mediated suppression of ERK1/2 phosphorylation was abrogated in these cells (supplemental Figure IA). Furthermore, PPARγ activation was suppressed in the cells overexpressed with constitutively active MEKK, and nifedipine-induced PPARγ activation was also abrogated in these cells (supplemental Figure IB). These results suggest that the suppression of ERK1/2 by nifedipine is mainly involved in nifedipine-induced PPARγ activation.

Nifedipine Suppresses LPS-Induced MCP-1 Expression Through PPARγ Activation

LPS (1 μg/mL) induced the expression of MCP-1 mRNA and protein in macrophages, and this expression was partially suppressed by nifedipine (Figure 5A and 5B). Pretreatment with PPARγ siRNA, which suppressed the expression of PPARγ-Ser112 phosphorylation is involved in nifedipine-induced PPARγ activation. A, RAW264.7 cells were transfected with (AOX)3-luc and pCMX-PPARγ2 [wild type (WT)] or pCMX-PPARγ2-S112A (PPARγ2-S112A) and were incubated with medium A in the absence or presence of 1 mmol/L nifedipine (Nife) for 24 hours. Luciferase activity was measured by Dual-Luciferase Reporter Gene Assay system. The data represent the mean±SEM of 5 separate experiments. *P<0.01 vs control. B, Mouse peritoneal macrophages were incubated with medium A in the absence or presence of indicated concentration of nifedipine for 24 hours. Protein samples were immunoblotted with anti-phospho-PPARγ2 or anti-PPARγ2 antibodies. The data are representative of 4 experiments. C, RAW264.7 cells were transfected with pCMX-PPARγ2 or mock, and then cells were incubated with medium A in the absence or presence of 1 mmol/L nifedipine for 24 hours. Protein samples were immunoblotted with anti-phospho-PPARγ2 or anti-PPARγ2 antibodies. The data are representative of 4 experiments. RLU indicates relative light unit.
PPARγ1 and PPARγ2 by 76 and 82%, respectively (Figure 5C), restored the nifedipine-mediated suppression of MCP-1 mRNA and protein expression (Figure 5A and 5B). Moreover, U0126, as well as nifedipine, suppressed LPS-induced MCP-1 mRNA expression, and pretreatment with PPARγ siRNA restored these effects (supplemental Figure IIA). These results suggest that nifedipine suppresses MCP-1 expression through inactivation of ERK1/2 and subsequently activation of PPARγ.

Other reports indicate that MCP-1 expression is mediated by nuclear factor κB (NF-κB) activation. In an NF-κB-Luc luciferase assay system, LPS (1 μg/mL) increased NF-κB activity and nifedipine blocked this activation (Figure 5D). Treatment with siRNA for PPARγ restored nifedipine-mediated suppression of NF-κB activity (Figure 5D).

**Nifedipine Induces ABCA1 Expression Through PPARγ Activation**

The ABCA1, which is involved in the control of apolipoprotein-mediated cholesterol efflux, is induced by PPARγ activation. Here, nifedipine induced ABCA1 mRNA expression, as detected by real-time RT-PCR (Figure 5E), and this effect could be blocked by PPARγ siRNA (Figure 5E). Western blot analysis also revealed that nifedipine increased ABCA1 protein expression (Figure 5F), and this effect was suppressed by PPARγ siRNA (Figure 5F). Moreover, U0126 also induced ABCA1 mRNA expression, and this effect was suppressed by pretreatment with PPARγ siRNA (supplemental Figure IIB). We further examined the effect of nifedipine on oxidized LDL-induced foam cell formation and accumulation of cholesteryl esters in macrophages. Our results show that nifedipine had no effect on oxidized LDL-induced foam cell formation (supplemental Figure IIIA) and increase in the accumulation of cholesteryl esters (supplemental Figure IIIB) in macrophages.

**Nifedipine Suppresses the Progression of Atherosclerosis in apoE−/− Mice through PPARγ Activation**

Nifedipine treatment did not change body weight, systolic blood pressure, diastolic blood pressure, serum total cholesterol, triglyceride, or high-density lipoprotein cholesterol levels in apoE−/− mice (supplemental Table I). However, nifedipine decreased the Oil Red O staining of atherosclerotic lesion in the aortic sinus (Figure 6A and 6B) and whole aorta (Figure 6A and 6C). Western blot analysis of the aorta
revealed that nifedipine decreased phosphorylation of both PPARγ and ERK1/2 (Figure 6D), and nifedipine also increased transcription of PPARγ in the aorta (Figure 6E). Nifedipine also blocked MCP-1 mRNA expression in the aortic sinus of apoE−/− mice (Figure 6F) but increased ABCA1 mRNA (Figure 6G) and protein (Figure 6H) levels.

**Discussion**

Several in vivo\textsuperscript{18,19} and clinical\textsuperscript{20–24} studies have indicated that nifedipine attenuates the formation of atherosclerotic lesions. However, the underlying mechanism has not been defined. Here, we revealed for the first time that nifedipine can induce PPARγ activation in macrophages between 1 pmol/L and 10 nmol/L, and the therapeutic concentrations of nifedipine (40 nmol/L) and amlodipine (3 nmol/L) were close to the concentrations of CCBs in vitro study. Therefore, it is possible that nifedipine-mediated PPARγ activation appears to have potential physiological relevance. Moreover, nifedipine could enhance pioglitazone or ciglitazone-induced PPARγ activation. Thus, combination therapy with nifedipine and thiazolidinediones may help prevent atherosclerotic vascular diseases. Interestingly, amlodipine did not increase PPARγ activity, indicating that PPARγ activation is not a common effect of L-type CCBs. In addition, high-dose amlodipine and BATPA-AM reduced PPARγ activity, suggesting that decreases in intracellular calcium may mediate the suppression of PPARγ activity in macrophages. Our preliminary data indicated that nifedipine suppressed oxidized LDL-induced intracellular reactive oxygen species generation in macrophages, and this effect by nifedipine was higher than amlodipine (T. Matsumura et al, unpublished data). Moreover, one of the antioxidant reagents, N-(2-mercapto-procinyl)-glycine (NMPG) suppressed the phosphorylation of ERK1/2 and activated PPARγ transcripational activity assay, respectively. Data represent the mean±SEM of 5 separate experiments. *P<0.01 vs control mice.
Nifedipine increased luciferase activity by full-length PPARγ but not by PPARγ GAL4 chimera, indicating that nifedipine is not a direct PPARγ ligand and does not increase intracellular natural PPARγ ligands.

ERK1/2 is an important protein kinase in the progression of atherosclerosis. It has been reported that activating ERK1/2 phosphorylates PPARγ and downregulated its activity. Specific inhibition of ERK1/2 suppressed PPARγ phosphorylation and increased PPARγ activity. In addition, nifedipine decreased PPARγ phosphorylation, but this was blocked in a mutant (S112A) PPARγ construct. Nifedipine also decreased ERK1/2 phosphorylation, which was also reported in αT3-1 cells and rat vascular SMCs, and constitutive activation of ERK1/2 abrogated nifedipine-induced PPARγ activation. These results suggested that the suppression of ERK1/2 by nifedipine is mainly involved in nifedipine-induced PPARγ activation. However, further studies are needed to clarify how nifedipine inhibits ERK1/2.

MCP-1 plays a critical role in recruiting monocytes into early atherosclerotic lesions, because overexpression of MCP-1 in vessel wall macrophages led to increased foam cell formation and increased atherosclerosis. Moreover, deletion of MCP-1 attenuated the progression of atherosclerosis in atherosclerotic models in mice. Nifedipine suppresses angiotensin-II-induced MCP-1 expression through inhibiting NF-κB in rat vascular SMCs. Here, nifedipine suppressed LPS-induced MCP-1 expression and NF-κB activation in macrophages, and siRNA for PPARγ attenuated these effects. Moreover, inactivation of ERK1/2 also suppressed LPS-induced MCP-1 expression, and siRNA for PPARγ attenuated these effects. Therefore, nifedipine may suppress NF-κB-dependent MCP-1 expression through inactivation of ERK1/2 and subsequently PPARγ activation.

Nifedipine reduces atherosclerotic lesion areas in cholesterol-fed rabbits. Here, nifedipine suppressed the progression of atherosclerosis in apoE−/− mice, decreased phosphorylation of ERK1/2 and PPARγ, and activated PPARγ in the aortic sinus. Because PPARγ activators suppress the development of atherosclerosis in atherosclerotic models in mice, PPARγ activation may be critical for nifedipine-mediated suppression of atherosclerosis.

ABCA1 is a member of the ABC transporter family and is involved in the control of apoA1-mediated cholesterol efflux from macrophages. The activation of PPARγ induces ABCA1 expression in macrophages. We revealed for the first time that nifedipine, as well as U0126, induced ABCA1 mRNA expression, and this effect was abrogated by siRNAs for PPARγ, suggesting that nifedipine works via inactivation of ERK1/2 and subsequent PPARγ activation. Nifedipine significantly suppresses the acceleration of atherosclerosis and decreases cholesterol levels in aortic tissue in cholesterol-fed rabbits. These changes may be mediated by PPARγ-dependent increases in ABCA1 expression.

PPARγ agonists, including pioglitazone and rosiglitazone, improve insulin sensitivity and are now used for the treatment of type 2 diabetes. Because nifedipine can also activate PPARγ, treatment with nifedipine may improve insulin sensitivity. In fact, nifedipine can increase insulin sensitivity in spontaneously hypertensive rats and diabetic rats. Treatment with long-acting nifedipine improved insulin sensitivity in patients with hypertension but did not affect insulin sensitivity in hypertensive obese patients. Further research is necessary to determine the effectiveness of nifedipine on insulin resistance and diabetes.

In the present study, we revealed that the expression of PPARγ1 and PPARγ2 was detected in RAW264.7 cells by Western blot analysis. Moreover, we have reported that PPARγ mRNA was also expressed in RAW264.7 cells by real-time RT-PCR assay. On the other hand, we revealed that PPARγ agonists did not increase the luciferase activity in RAW264.7 cells transfected with (AOX)3-luc plasmid alone, but they increased it in cells cotransfected with pCMV-PPARγ2 and (AOX)3-luc plasmid. These results suggested that although the proteins were expressed in RAW264.7 cells, these proteins lack the transcriptional activity on the promoter that possesses peroxisome proliferator response element sequence. This may be due to the expression of abnormal PPARγ1 and PPARγ2 protein, which may lack ligand-binding activity or DNA-binding activity in RAW264.7 cells.

In conclusion, nifedipine, unlike amlodipine, activated PPARγ by decreasing phosphorylation of PPARγ, thereby suppressing MCP-1 expression and inducing ABCA1 expression in macrophages. Moreover, nifedipine suppressed the acceleration of atherosclerosis in apoE−/− mice. These results could explain, at least in part, the effectiveness of nifedipine, unlike amlodipine, in hypertensive patients as well as in patients with diabetes and hyperlipidemia that have athero-sclerotic vascular diseases.

Acknowledgments

We thank Dr Michael Brownlee and Dr Yusuke Takeuchi for plasmids and helpful advice and Kenshi Ichinose from our laboratory for helpful advice and assistance.

Sources of Funding

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Japan, 21591144 (to T.M.), a grant from the Takeda Science Foundation (T.M.), and the Advanced Education Program for Integrated Clinical, Basic, and Social Medicine, Graduate School of Medical Sciences, Kumamoto University (Support Program for Improving Graduate School Education, Ministry of Education, Culture, Sports, Science, and Technology, Japan).

Disclosures

None.

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Arterioscler Thromb Vasc Biol. 2010;30:1598-1605; originally published online May 27, 2010; doi: 10.1161/ATVBAHA.109.202309

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Table I

Body weight (BW), blood pressure (BP), plasma lipid profile and plasma MCP-1 level in control or nifedipine-treated apoE–/– mice

<table>
<thead>
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<th></th>
<th>control (n=9)</th>
<th>nifedipine (n=10)</th>
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<tr>
<td>BW (g)</td>
<td>36.2 ± 2.1</td>
<td>37.7 ± 2.8</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>111 ± 12</td>
<td>117 ± 8</td>
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<tr>
<td>DBP (mmHg)</td>
<td>77 ± 6</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>1013 ± 82</td>
<td>1049 ± 55</td>
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<tr>
<td>TG (mg/dl)</td>
<td>166 ± 52</td>
<td>182 ± 48</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>35 ± 7</td>
<td>37 ± 6</td>
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SBP indicates systolic BP; DBP, diastolic BP; TC, total cholesterol; TG, triglycerides; and HDL-C, HDL cholesterol.
Supplemental figure I

Constitutive activation of ERK1/2 ameliorates nifedipine-induced PPARγ activation.

RAW264.7 cells were transfected with CA-MEKK plasmid, which is an expression plasmid for constitutively activate MEKK, or mock plasmid, and were cultured with medium A for 4 h. Cells were incubated with or without 100 pmol/L nifedipine for 24 h. A, protein samples were immunoblotted with anti-phospho-ERK1/2 or anti-ERK1/2 antibodies. The data are representative of four experiments. B, PPARγ activity was determined using the full-length PPARγ luciferase assay system. Data represent the mean ± SEM of four separate experiments. *, p<0.01, vs. control cells transfected with mock.
Supplemental figure II
Anti-atherosclerotic effect of nifedipine through PPARγ activation.

Mouse peritoneal macrophages were transfected with siRNA of control (con) or PPARγ, and were cultured with medium A for 4 h. Cells were incubated with or without 1 μmol/L U0126. After 24 h incubation, the cells were incubated with or without 1 μg/mL LPS for 3 h. MCP-1 mRNA level (A) and ABCA1 mRNA level (B) were determined by real-time RT-PCR. Data represent the mean ± SEM of four separate experiments. *, p<0.01, vs. control. **, p<0.01, vs. cells with LPS alone. #, p<0.01, vs. cells with PPARγ siRNA alone.
Supplemental figure III

Effect of nifedipine on cholesteryl esters accumulation by oxidized-low density lipoprotein (Ox-LDL).

Mouse peritoneal macrophages were incubated with medium A in the absence or presence of 20 µg/mL Ox-LDL and/or 1 nmol/L nifedipine (Nife) for 48h. (A), Oil-Red O staining. (B), Cellular lipids were extracted and determination of free cholesterol and cholesteryl esters was performed as described in supplemental methods. Data represent the mean of three separate experiments. *, p<0.01, vs. the control.
Supplement Material

Supplemental Methods.

Materials

PD98059 and ciglitazone were purchased from Calbiochem (San Diego, CA, USA). Nifedipine, fenofibrate, bezafibrate, and pioglitazone were kindly given from Bayer Yakuhin, Ltd. (Osaka, Japan), Kaken Pharmaceutical Co., Ltd (Tokyo, Japan), Kissei Pharmaceutical Co., Ltd (Nagano, Japan), and Takeda Pharmaceutical (Osaka, Japan), respectively. Amlodipine, BAPTA-AM (1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxy-methyl ester) and U0126 were purchased from BIOMOL International, L.P. (Plymouth Meeting, PA, USA). LPS (Escherichia coli. O111:B4) was purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal anti-ERK1/2, anti-PPARγ, anti-PPARγ2 and anti-phospho PPARγ2 antibodies, and goat polyclonal anti-β-actin antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal anti-phospho ERK1/2 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal anti-ABCA1 antibody was purchased from Novus Biologicals, Inc. (Littleton, CO, USA). All other chemicals were of the best grade available from commercial sources.

Animals

All animal procedures were approved by the Animal Research Committee at Kumamoto University, and all procedures conformed to the Guide for the Care and Use of Laboratory Animals issued by the Institute of Laboratory Animal Resources. ApoE−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA), and were maintained on the C57BL/6 background strain. Mice were given access to food and water ad libitum in the Animal
Supplement Material

Resource Facility at Kumamoto University under specific pathogen-free conditions. The diet was a normal rodent chow diet for mouse (CLEA, Tokyo, Japan). Twenty male mice, 6 weeks old, were treated with nifedipine (10 mg/kg/day) or placebo (control) by oral administration. After 10 weeks, they were sacrificed, and the aortic sinus was used for Western blot and PPARγ transcription assays as described below. Six µm-thick frozen sections of aortic sinus obtained from ApoE−/− mice were stained with oil red O. Digital microphotographs of the aortic sinus were analyzed for lesion size in specific regions by finding the stained surface area using ImageJ (NIH). Blood pressure was periodically determined by the tail-cuff method using a photoelectric tail-cuff detection system (model BP-98A, Softron, Tokyo, Japan).

Concentrations of plasma total cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol were performed commercially by Skylight Biotech Inc, (Akita, Japan).

Full-length PPARγ luciferase assay system

The reporter plasmid (AOX)³-luc, containing three copies of an upstream activating sequence for the PPAR response element of acyl-coenzyme A oxidase and a thymidine kinase gene promoter (tk-promoter) in front of a luciferase cDNA, and PPARγ2 expression plasmid (pCMX-PPARγ2), containing the mouse PPARγ2 gene driven by the CMV promoter, were described previously. To measure the PPARα or PPARγ activity, RAW264.7 cells, mouse peritoneal macrophages, or THP-1 macrophages (2×10⁶ cells/well) were transfected with (AOX)³-luc, and pCMV-hPPARα or pCMX-PPARγ2 using Lipofectamine 2000 (Invitrogen). The luciferase assay was performed as described below.

PPARγ GAL4 chimera assay system

The fusion protein expression vector, pM-PPARγ, containing residues 1-147 of the GAL4 DNA-binding domain and 204-505 of the human PPARγ ligand-binding domain, driven by
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The reporter plasmid, p4xUASg-tk-luc, containing four copies of a 17-mer upstream activating sequence for the GAL4 DNA-binding domain and a thymidine kinase gene promoter (tk-promoter) in front of a luciferase cDNA, was also described previously. To measure the PPARγ ligand-binding activity, RAW264.7 cells (2×10^6 cells/well) were transfected with p4xUASg-tk-luc and pM-PPARγ, and subjected to the luciferase assays as described below.

Luciferase assays

pCMX-PPARγ2-S112A, which has a mutation of Ser112 to Ala within the putative ERK1/2 site in mouse PPARγ2, was kindly given from Dr. Michael Brownlee (Departments of Medicine and Pathology, Albert Einstein College of Medicine). NF-κB luciferase reporter plasmid (pNFkB-Luc) was purchased from Takara Bio Inc. (Siga, Japan). Luciferase assay was performed essentially using a Dual-Luciferase Reporter Gene Assay system (Promega) as previously reported.

Transcriptional activity of PPARγ

Transcription activity of PPARγ was assayed using an enzyme-linked immunosorbent assay-based PPARα, δ, γ Complete Transcription Factor Assay Kit (Cayman Chemical., Ann Arbor, MI, USA) according to the manufacturer's instructions as previously described.

Transfection of siRNA and plasmids

The siRNA against PPARγ and an unrelated 21-nucleotide siRNA duplex as a control were purchased from Santa Cruz Biotechnology. An expression vector for constitutively active mitogen-activated protein kinase (MAPK) kinase kinase (MEKK) (pFc-MEKK), which encodes amino acids 380-672 of MEKK, lacks the regulatory NH2-terminal domain and
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activates MAPK pathway, was kindly gifted from Dr. Yusuke Takeuchi (Department of Pharmacology, Tohoku University Graduate School of Pharmaceutical Sciences). Mouse peritoneal macrophages (2×10^6 cells/well) or RAW264.7 cells (2×10^6 cells/well) were transfected with PARγ siRNA, control siRNA, pFc-MEKK or mock using Lipofectamine 2000 (Invitrogen). After 4 h incubation, real-time RT-PCR, enzyme-linked immunosorbent assay (ELISA), Western blot assay and luciferase assay were performed.1,2 PPARγ siRNA inhibition of PPARγ expression was tested by Western blot analysis as described below.

Adenoviral Vectors

Mouse peritoneal macrophages (2×10^6 cells/well) were infected with a recombinant replication-deficient adenovirus containing a gene of dominant negative ERK (DN-ERK) for 48 h at the multiplicity of infection (MOI) of approximately 50, as described previously,2,5 and were cultured with medium A for 3 h. This condition conferred expression of LacZ as a marker gene in nearly 100% of transfected cells.2,5

Western blot analysis

RAW264.7 cells (2×10^6 cells/well) or mouse peritoneal macrophages (2×10^6 cells/well) were incubated with indicated effectors, and whole cell lysates were purified as described previously.6 Protein concentrations were determined by the Micro BCA Protein Assay Reagent (Pierce Chemical Co.). Samples were applied to 10% SDS gels, separated by electrophoresis, and then transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA). Membranes were incubated with appropriate antibodies at a dilution of 1:1000 for 2 h. After washing, the membranes were stained with horseradish peroxidase conjugated goat anti-rabbit antibodies (Santa Cruz, Inc.).8 Immunoreactive bands were quantified by the National Institute of Health (NIH) image analysis software.6
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Real-time RT-PCR analysis

Real-time RT-PCR analysis was performed essentially as previously reported.\textsuperscript{1,2,4} Specific primers for mouse MCP-1, ABCA1 and 36B4 were as follows: MCP-1, forward primer, 5’-GGTCCCTGTCATGCTTCT-3’ and reverse primer, 5’-CATCTTGCTGGTGAATGAGT-3’; ABCA1, forward primer, 5’-GCCAACCTGCAGAAGGGAGA-3’ and reverse primer, 5’-GCTCACACGCTCCTGGATCA-3’; 36B4, forward primer, 5’-CTGATCCATCTGCATTTCG-3’ and reverse primer, 5’-TCCTCATCTGATTCCTCGACT-3’. Quantitative results for MCP-1 and ABCA1 were normalized by the levels of 36B4 mRNA.

Enzyme-linked immunosorbent assay (ELISA) for MCP-1

Mouse peritoneal macrophages (2×10\textsuperscript{6} cells) were cultured with the indicated effectors for 1 h, and then 1 µg/mL of LPS was added. After 3 h incubation, the media was collected and the concentration of MCP-1 protein was determined using Mouse MCP-1 ELISA assay kit (Invitrogen).\textsuperscript{1}

Lipoprotein preparation

Human low density lipoprotein (LDL) (d=1.019 to 1.063 g/mL) was isolated by ultracentrifugation from the plasma of consented normolipidemic subjects obtained after overnight fasting.\textsuperscript{7} LDL was dialyzed against 0.15 mol/L NaCl and 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4. oxidized LDL (Ox-LDL) was prepared by incubation of LDL with 5 µmol/L CuSO\textsubscript{4} for 20 h at 37°C followed by the addition of 1 mmol/L EDTA and cooling.\textsuperscript{7} The concentration of proteins was determined by BCA protein
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assay reagent (Pierce Chemical Co., Rockford, IL). Endotoxin level of Ox-LDL was <1 pg/µg protein measured by Toxicolor system (Seikagaku Corp, Japan).7

Determination of cellular cholesterol content

Macrophage monolayers (3 x 10^6 cells) were incubated with 20 µg/mL of Ox-LDL for 48 h in the absence or presence of 1 nmol/L of nifedipine. After incubation, cellular lipids were extracted and both total cholesterol and free cholesterol were quantified using a modified enzymatic fluorometric method.8 The level of cholesteryl esters was calculated by subtracting free cholesterol from total cholesterol.

Statistical analysis

All data are expressed as the mean ± SEM. Statistical differences between groups were examined by one-factor ANOVA. P<0.01 was statistically significant.

References


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