Nifedipine Activates PPARγ and Exerts Antioxidative Action Through Cu/ZnSOD Independent of Blood-pressure Lowering in SHRSP

Ryo Hashimoto¹, Seiji Umemoto², Fengling Guo³, Kyoko Umeji¹, Shinichi Itoh¹, Hiroko Kishi³, Sei Kobayashi³, and Masunori Matsuzaki¹

¹Department of Medicine and Clinical Science, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan
²Pharmaceutical Clinical Research Center, Yamaguchi University Hospital, Yamaguchi, Japan
³Department of Molecular Physiology and Medical Bioregulation, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan

Aim: It has been shown that the calcium antagonist nifedipine upregulates superoxide dismutase (SOD). Although the peroxisome proliferator-activated receptor (PPAR) response element is located in the promoter region of Cu/ZnSOD, it remains unclear whether nifedipine upregulates PPARs and inhibits vascular remodeling. We hypothesized that nifedipine activates PPARγ, inhibits vascular remodeling, and improves vascular function in hypertension.

Methods: Stroke-prone spontaneously hypertensive rats (SHRSP) were treated with vehicle, nifedipine, and PPARγ selective antagonist GW9662 with nifedipine.

Results: Systolic blood pressure in the three SHRSP groups was higher (p<0.01), and the left ventricular weight/body weight ratio was greater (p<0.01) than in the Wistar-Kyoto rat (WKY) group with no differences observed among the three SHRSP groups. In the SHRSP heart, nifedipine significantly inhibited intramyocardial arterial remodeling and perivascular fibrosis, and reduced oxidative stress, while it significantly restored adiponectin and the smooth muscle cell (SMC) phenotype, and selectively restored PPARγ and Cu/ZnSOD expression/activities to their levels in the WKY rat heart. Furthermore, nifedipine induced a dose-dependent increase in PPARγ expression in cultured vascular SMCs. These effects of nifedipine were completely abolished by the co-administration of GW9662 with nifedipine. Nifedipine treatment significantly improved acetylcholine-induced relaxation by 27% compared with the vehicle SHRSP group, but it was still significantly impaired by 20% compared with the WKY group.

Conclusions: Nifedipine may inhibit vascular remodeling and improve vascular function by selective activation of PPARγ through the activation of Cu/ZnSOD in hypertension.


Key words: Hypertension, Vascular remodeling, Calcium channel blocker, Superoxide dismutase, Peroxisome proliferator-activated receptor

Introduction

Oxidative stress induced by reactive oxygen species (ROS), such as superoxide (·O2⁻), plays an important role in atherogenesis, and the redox state is determined by the balance between antioxidants and the ROS generating system, and the imbalance between vascular antioxidant and oxidant systems might play an important role in coronary atherogenesis. One primary cellular defense against ·O2⁻ is superoxide dismutase (SOD), and the predominant activity of SOD in the vasculature is attributed to Cu/ZnSOD, which may play an important role in preventing vascular dysfunction and atherosclerosis in hypertension.

Peroxisome proliferator-activated receptors
(PPARs) heterodimerize with retinoid-X receptors (RXRs) and modulate the functions of many target genes present in the endothelium, vascular smooth muscle cells (SMCs), and heart. Recent evidence indicates that PPARα and PPARγ exert cardiovascular protective effects independent of their metabolic actions. In addition, it has been reported that the PPAR response element (PPRE) is located in the promoter region of Cu/ZnSOD genes, which may contribute to the antiatherogenic effects of PPARs within the vasculature.

The primary pharmacodynamic effect of the dihydropyridine calcium antagonist nifedipine is the dilatation of both large and small arteries through a reduction in vascular smooth muscle (SM) contraction in the arterial wall. Furthermore, nifedipine shows antiatherogenic properties, slows the progression of atherosclerosis, and shows some inhibition of new-onset diabetes in hypertension, independent of its blood pressure-lowering effect. It is also reported that nifedipine indirectly upregulates MnSOD. Taken together, these results suggest that nifedipine may inhibit the progression of atherosclerosis through antioxidant mechanisms via PPARs in hypertension; however, it remains unclear whether nifedipine upregulates SODs and PPARs in hypertension in vivo.

Aim

In this study, we hypothesized that nifedipine may inhibit vascular remodeling and improve vascular function in hypertension by modulating oxidative stress through the activation of Cu/ZnSOD via the PPARγ pathway.

Methods

Animals and Experimental Protocol

The Ethics Committee for Animal Experimentation at Yamaguchi University School of Medicine approved the experimental protocol used in this study.

To examine the effects of nifedipine on vascular remodeling, PPARs, and antioxidant systems independent of blood pressure lowering, we used subpressor-dose nifedipine in the study. The doses used in the experiments were determined by preliminary experiments. Twelve-week-old male Wister-Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP) were obtained from Charles River Japan (Yokohama, Japan). WKY were treated with a vehicle (WKY group; n = 20), and SHRSP were treated into three groups of 20 rats each and treated for 4 weeks with a vehicle (SHRSP group; n = 20), nifedipine (1 mg·kg⁻¹·day⁻¹, nifedipine group; n = 20), or PPARγ selective antagonist GW9662 (1 mg·kg⁻¹·day⁻¹, nifedipine + GW9662 group; n = 20), in addition to nifedipine (1 mg·kg⁻¹·day⁻¹). All rats received 1% NaCl in drinking water, and nifedipine and GW9662 were given subcutaneously by osmotic minipumps (model 2ML2; ALZET Osmotic Pumps, Cupertino, CA). Without anesthetizing the rats, we determined their systolic blood pressures (SBPs) by tail-cuff plethysmography.

After the 4-week treatment period, the rats were weighed and euthanized with a sodium pentobarbital overdose; the hearts were then excised and weighed. Some of the excised hearts were perfused and fixed for 5 min at a pressure of 90 mmHg with heparinized saline followed by Bouin’s solution, and then paraffin-embedded to obtain 4-µm-thick sections those were stained with Sirius red for histological analysis. Other heart-tissue sections were snap-frozen with optimal cutting temperature (O.C.T.) compound in liquid nitrogen to obtain fresh-frozen, 30-µm-thick sections for dihydroethidine (DHE) and hydrogen peroxide (H₂O₂), 5-(and6-)chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate, acetyl ester (DCF-DA) staining. The left ventricles of other hearts were separated, washed with heparinized saline, frozen in liquid nitrogen, and stored at −80°C until they were used for immunoblotting and for ROS-related enzyme and PPAR assays. To avoid contaminating the epicardial large coronary arteries as much as possible, we did not use the remaining base-side heart tissues for the study.

Chemicals and Antibodies

Nifedipine was provided by Bayer Yakuhin, Ltd. (Tokyo, Japan). PPARγ selective antagonist GW9662 (2-chloro-5-nitrobenzanilide) was purchased from SIGMA (St. Louis, MO). The following were used for immunobots: mouse monoclonal antibodies against human α-tubulin (DAKO Japan, Kyoto, Japan), human endothelial nitric oxide synthase (eNOS) (BD Transduction Laboratories, San Diego, CA), human PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA), mouse PPARα (Affinity Bioreagents, Golden, CO), rabbit contractile-type SM myosin heavy chain isoform SM2 (Yamasa, Choshi, Japan), rat adiponectin (CHEMICON International, Temecula, CA), goat polyclonal antibody against human Cu/ZnSOD, human p22phox and human p47phox components of NAD(P)H oxidase (Santa Cruz Biotechnology), and rabbit polyclonal antibody against human RXR (Santa Cruz Biotechnology).
Cell Culture and Cell-culture Experiments

Rat aortic SMCs were purchased from Cascade Biologics, Inc. (Portland, OR), and cultured in SM basal medium (Clonetics, Walkersville, MD) and 5% fetal bovine serum as previously reported\(^\text{10}\). Experiments were performed with 0.5% serum and no additives. Harvested vascular SMCs were treated in 10 µL/10\(^6\) cells of lysis buffer (1% sodium dodecyl sulfate (SDS), 100 mmol/L NaCl, 50 mmol/L Tris-HCl [pH 8.0], 20 mmol/L EDTA) and stored at −80°C until used for immunoblotting. Samples containing 20 µg protein each were separated by SDS-15% polyacrylamide gel electrophoresis (PAGE). Blots were blocked in 5% skim milk in PBS for 1 hour, treated with the antibody to PPAR\(_\gamma\) at 4°C overnight, and then incubated with the peroxidase-conjugated secondary antibody for 1 hour. Protein expression was determined with an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK) using NIH Image. The blot was then reprobed with α-tubulin to confirm equal protein loading in each well.

Histological Analysis

Fixed specimens were paraffin-embedded by conventional procedures, and then sectioned into 4-µm slices and stained with Sirius red. In each heart, the wall-to-lumen ratio of 10 intramyocardial arteries < 150 µm in diameter, as well as the severity of perivascular fibrosis and collagen deposition in the heart, which was representative of collagen, including types I and III, were identified using birefringency under polarized light illumination. These parameters were then evaluated using a computer-assisted image analysis system with NIH Image 1.62 as previously reported\(^\text{17}\), and the mean values for each heart were used for statistical analysis.

Immunoblotting

Rat heart tissue was homogenized, and α-tubulin, Cu/ZnSOD, adiponectin, which is expressed in the heart\(^\text{18, 19}\), and has the PPRE sequence (5′-AAC\,...\,TGGTCAAGGTCA-3′) in its gene just as Cu/ZnSOD does, RXR, PPAR\(_\alpha\), PPAR\(_\gamma\), \(p^{22}\text{phox}\) and \(p^{47}\text{phox}\) were separated by SDS-15% PAGE; SM2, calponin, and eNOS were separated by SDS-10% PAGE. Primary antibodies against α-tubulin, Cu/ZnSOD, RXR, \(p^{22}\text{phox}\), \(p^{47}\text{phox}\), SM2 and calponin were used at a dilution of 1:500; PPAR\(_\alpha\), PPAR\(_\gamma\) and eNOS were used at a dilution of 1:1000; adiponectin was used at a dilution of 1:2000. Protein expression was determined with an enhanced chemiluminescence system (Amersham Biosciences) using NIH Image, as described previously\(^\text{16}\).

In Situ Evaluation of Superoxide Content

For \textit{in situ} imaging of ROS, we incubated frozen sections (30 µm) of rat heart tissues with fluorophores sensitive to -O\(_2^−\), DHE 10 µmol/L\(^\text{21}\), or DCF-DA 10 µmol/L (Invitrogen Corporation, Carlsbad, CA), since H\(_2\)O\(_2\) is also known to contribute to oxidative damage. Images of DHE and DCF were obtained with a laser scanning confocal microscope (LSM510; Carl Zeiss, Jena, Germany). DHE specifically reacts with intracellular -O\(_2^−\) and is converted to the red fluorescent compound ethidium, which then binds irreversibly to double-stranded DNA and appears as punctate nuclear staining. These data were evaluated in a blind fashion using a computer-assisted image analysis system with NIH Image 1.62 as previously reported\(^\text{17}\), and the mean value for each heart was used for statistical analysis and expressed as a percentage of the corresponding data for the WKY group. The specificity of DHE and DCF signals for -O\(_2^−\) and H\(_2\)O\(_2\) detection, respectively, was confirmed by preincubation with polyethylene glycol-superoxide dismutase (PEG-SOD; 500 U/mL, SIGMA) and PEG-catalase (350 U/mL, SIGMA), respectively\(^{\text{22, 23}}\).

Measurement of SOD, Catalase, and Glutathione Peroxidase Activities

All assays were performed at 37°C and under ambient oxygen pressure. SOD activities were determined based on the SOD-mediated increase in the autoxidation rate of 5,6,6a, 11b-tetrahydro-3,9,10-trihydroxybenzo[\(c\)]fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm with a spectrophotometric assay as previously reported\(^\text{21, 23}\). Briefly, heart tissues were washed 0.9% NaCl containing heparin to remove red blood cells, followed by homogenization in 50 mmol/L potassium phosphate (pH 7.4) containing 0.3 mol/L KBr and a cocktail of protease inhibitors. The homogenates were sonicated and extracted at 4°C for 15 min. Next, 40 µL of tissue homogenate was added to 900 µL of 2-amino-2-methyl-1,3-propanediol, containing boric acid and diethylenetriaminepentaacetic acid (DTPA) (pH 8.8), and then 30 µL of 1,4,6-trimethyl-2-2 vinylpyridinium trifluoromethanesulfonate in HCl was added, briefly vortexed, and incubated at 37°C for 1 min. We added 30 µL of 5,6,6a, 11b-tetrahydro-3,9,10-trihydroxybenzo[\(c\)]fluorene, in HCl containing DTPA and ethanol, and immediately measured the absorbance at 525 nm spectrophotometrically. SOD activity based on the ratio of the autoxidation rates in the presence and absence of SOD was measured using an SOD-525TM reagent kit (OXIS).
Table 1. Systolic blood pressure, body weight and left ventricular weight in 16-week-old rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY</th>
<th>SHRSP</th>
<th>Nifedipine</th>
<th>Nifedipine+GW9662</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>112 ± 3</td>
<td>195 ± 3*</td>
<td>200 ± 4*</td>
<td>204 ± 2*</td>
</tr>
<tr>
<td>BW, g</td>
<td>319 ± 12</td>
<td>276 ± 9*</td>
<td>275 ± 6*</td>
<td>266 ± 4*</td>
</tr>
<tr>
<td>LVW, mg</td>
<td>1.070 ± 54</td>
<td>1.130 ± 53</td>
<td>1.119 ± 24</td>
<td>1.142 ± 39</td>
</tr>
<tr>
<td>LVW/BW, mg/g</td>
<td>3.49 ± 0.1</td>
<td>4.11 ± 0.2*</td>
<td>4.07 ± 0.1*</td>
<td>4.30 ± 0.2*</td>
</tr>
</tbody>
</table>

WKY were treated with vehicle; SHRSP were treated with vehicle, nifedipine (1 mg·kg⁻¹·day⁻¹), or nifedipine (1 mg·kg⁻¹·day⁻¹) and GW9662 (1 mg·kg⁻¹·day⁻¹) for 4 weeks. Values are means ± SEM. *p < 0.01 versus the WKY group. SBP = systolic blood pressure, BW = body weight, LVW = left ventricular weight. Experiments, n = 10.

International, Foster, CA), as previously reported. Absolute ethanol/chloroform, 62.5/37.5 (v/v), was used to inactivate MnSOD and specifically measure Cu/ZnSOD activity according to the manufacturer's recommendations.

The activity of catalase in rat heart tissue homogenate was assessed using a Catalase-520™ reagent kit (OXIS International), and the activity of glutathione peroxidase (GPx) in rat heart tissue was assessed indirectly from the oxidation of NAD(P)H to NADP⁺ using a GPx-340™ reagent kit (OXIS International), according to the manufacturer's recommendations.

PPARγ Activity

PPARγ activation was assayed using an ELISA-based transactivation TransAM PPARγ kit (Active Motif, Carlsbad, CA) following the manufacturer's protocol. The TransAM PPARγ kit contains a 96-well plate with immobilized oligonucleotides containing a PPRE. PPARs contained in nuclear extracts bind specifically to this oligonucleotide and are detected through the use of an antibody directed against PPARγ. The horse-radish peroxidase (HRP)-conjugated secondary antibody provided a sensitive colorimetric readout that was quantified by spectrophotometry.

Endothelium-Dependent Relaxation in Mesenteric Arteries

Endothelium-dependent relaxation was induced by the application of acetylcholine (ACh) during the contraction induced by norepinephrine (NE), and was assessed in a blind fashion. The superior mesenteric artery (SMA) of each rat was excised and then placed in ice-cold physiological salt solution (PSS (mM): NaCl, 123; KCl, 4.7; NaHCO₃, 15.5; KH₂PO₄, 1.2; CaCl₂, 1.25; and D-glucose, 11.5). All solutions were gassed with a mixture of 95% O₂ and 5% CO₂ (pH adjusted to 7.4). Under a microscope, the adventitia was carefully decorticated with minimal stretch to the SM, and the endothelium was carefully kept. SM strips of rat SMA were manually dissected into small strips (width: 300–400 μm; length: 1–2 mm), and were then connected to an isometric force transducer (UL-2GR; Minebea, Tokyo, Japan) and mounted on a bubble plate made of acrylic resin. The solutions were heated at 37°C. Each strip was repeatedly exposed to 118 mM K⁺-PSS until the responses became stable. The strips were then contracted with 10⁻⁵ mol/L NE. After the contractions reached a steady level, the relaxant effects of ACh were studied by adding ACh in increasing concentrations, from 10⁻⁷ to 10⁻³ mol/L.

Statistical Analysis

All values are expressed as the means ± SEM. The experimental groups were compared using ANOVA followed by Scheffe’s multiple comparisons. A p value < 0.05 was considered to indicate statistical significance.

Results

Blood Pressure and Left Ventricular Weight

Throughout the experiments, the SBPs in the three SHRSP groups were significantly higher than in the WKY group, with no differences observed among the three SHRSP groups. Furthermore, left ventricular weight/body weight ratios in the three SHRSP groups were significantly greater than in the WKY group, with few differences observed among the three SHRSP groups (Table 1).

Effects of Nifedipine on Vascular Remodeling, Perivascular Fibrosis, Smooth Muscle Cell Phenotypes, and eNOS Expression in the SHRSP Heart

Fig. 1A shows that both the wall-to-lumen ratio of the intramyocardial artery and the perivascular collagen volume fraction in the vehicle SHRSP group were significantly greater than in the WKY group. Among the three SHRSP groups, nifedipine alone significantly reduced both the wall-to-lumen ratio and the perivascular collagen volume fraction compared with those in the vehicle SHRSP group, and the ratios
reached the same levels as those observed in the WKY group; however, GW9662 completely abolished the effects of nifedipine on the ratios in the SHRSP heart.

The expressions in the heart of both SM2 (Fig. 1B) and calponin (Fig. 1C), which are molecular markers for the contractile-type SMC phenotype, were significantly decreased in the vehicle SHRSP group compared with the WKY group. Nifedipine restored their expressions to the same levels as observed in the WKY group, but their expressions in the nifedipine + GW9662 group were similar to those in the vehicle SHRSP group. Furthermore, eNOS expression was 62% lower in the SHRSP heart than in the WKY heart, whereas nifedipine treatment with or without GW9662 had little effect on eNOS expression in the SHRSP heart (Fig. 1D).

Effects of Nifedipine on Oxidative Stress in the SHRSP Heart Vasculature

Fig. 2 shows the representative DHE and DCF staining and quantitative analysis of the -O2·− content in the rat heart. DHE and DCF densities in the hearts of the vehicle SHRSP group were markedly higher than in the WKY group. By co-incubation with either PEG-SOD or PEG-catalase, the increase in density in the vehicle SHRSP heart was almost completely abolished, a finding that provided specific evidence for the presence of increased ·O2·− or H2O2 in the SHRSP heart.

Nifedipine significantly suppressed the ·O2·− content in the SHRSP heart to the same level as that observed in the WKY group; however, GW9662 completely abolished the effects of nifedipine on the ·O2·− content in the SHRSP heart. The pattern of the H2O2-associated signal with nifedipine and GW9662 was similar to that of DHE staining in the SHRSP heart.

Effects of Nifedipine on SOD, Other Antioxidant Enzymes and NAD(P)H Oxidase in the SHRSP Heart

Cu/ZnSOD expression in the vehicle SHRSP group was significantly reduced by 67% compared with the WKY group. Nifedipine increased Cu/ZnSOD expression in the SHRSP heart to a level 2.8-fold higher than that in the vehicle SHRSP group, and the expres-
Effects of Nifedipine on PPARs, RXR, and Adiponectin in the SHRSP Heart

PPARγ expression and its activity in the vehicle SHRSP group were significantly reduced compared with the WKY group. Nifedipine increased PPARγ expression and activity, such that they reached the same levels as in the WKY group; however, the effects of nifedipine on PPARγ expression and activity in the SHRSP heart were completely abolished by the co-administration of GW9662 (Fig. 4A and 4B). The effect of nifedipine and GW9662 on adiponectin expression in the SHRSP heart was similar to the effect of nifedipine on PPARγ (Fig. 4C).

Similar to PPARγ, PPARα expression was also significantly reduced in the SHRSP heart compared with the WKY heart, while treatment with nifedipine and GW9662 had little effect on PPARα expression in the SHRSP heart (Fig. 4D). We also found very little effect of nifedipine and GW9662 on the expression of RXR, the heterodimeric partner of PPARγ, in the SHRSP heart (Fig. 4E).

Effects of Nifedipine on PPARγ Expression in Cultured Rat Smooth Muscle Cells

We further examined the effects of nifedipine on PPARγ expression in cultured rat aortic SMCs to confirm the direct activation of PPARγ by nifedipine. As shown in Fig. 5, nifedipine induced a dose-dependent increase in the expression of PPARγ, which was completely abolished with the co-administration of GW9662 with nifedipine.

Effects of Nifedipine on Endothelium-Dependent Relaxation in Superior Mesenteric Arteries

Finally, we examined the effects of nifedipine on endothelium-dependent relaxation in SMA. In SMA strips precontracted with $10^{-5}$ mol/L NE, ACh produced a dose-dependent relaxation in the WKY group (Fig. 6). In the vehicle SHRSP group, this relaxation was significantly impaired compared with that in the WKY group. Nifedipine treatment significantly improved ACh-induced relaxation by 27% compared with in the vehicle SHRSP group, but it was still significantly impaired by 20% compared with the WKY group, and the relaxation was almost completely abolished by the additional administration of GW9662 with nifedipine in SHRSP.

Discussion

As in our previous reports, although the re-
Production of Cu/ZnSOD activity was much greater among ROS-scavenging enzymes in the SHRSP heart, the selective restoration of Cu/ZnSOD by nifedipine in the SHRSP heart resulted in decreased ·O$_2^-$ content and the inhibition of vascular remodeling independent of blood-pressure lowering at the doses used. Furthermore, the co-administration of selective PPARγ antagonist GW9662 with nifedipine completely abolished the effects of nifedipine on Cu/ZnSOD in the SHRSP heart, suggesting that PPARγ may have been involved in the reduction of ·O$_2^-$ content in the SHRSP heart through Cu/ZnSOD activation. In contrast to previous reports$^{16, 29}$, in the present study, MnSOD activity and eNOS expression in the heart did not differ among the three SHRSP groups; the difference in experimental models between in vitro and our in vivo experimental conditions may explain this discrepancy. Furthermore, it has been reported that rat ecSOD is lacking in the vessel walls and is mainly present in plasma$^{30}$, and that ecSOD activity is also quite low in rat hearts$^{31}$, indicating the unlikelihood that ecSOD plays a critical role in the rat heart. Furthermore, in our study, the activities of other ROS-scavenging enzymes, GPx, and catalase, all of which may play an important role in the pathogenesis of atherosclerotic disease$^{32, 33}$, did not differ among the three SHRSP groups, a finding that suggests that these enzymes may be independent of the PPARγ-dependent pathway, and may not be centrally involved in the antiatherogenic properties of nifedipine in the SHRSP heart.

It was possible that the nifedipine-induced increase of Cu/ZnSOD activity in the SHRSP heart would enhance H$_2$O$_2$ production, which would induce oxidative damage in SHRSP$^2$. However, the in situ detection of H$_2$O$_2$ content in our study clearly
showed that the H$_2$O$_2$ content, similar to the ·O$_2^-$ content, was markedly decreased in the SHRSP heart by the administration of nifedipine, indicating that an increase in Cu/ZnSOD within the physiological range may not induce a pathological increase in H$_2$O$_2$ content, but instead may decrease H$_2$O$_2$ content in the SHRSP heart in vivo. These results suggest that Cu/ZnSOD might be critical for determining the level of ROS in the SHRSP heart in vivo.

It is reported that a local adiponectin system independent of serum adiponectin is present in the heart. In the study, we also demonstrated that adiponectin expression, like Cu/ZnSOD, was restored in the SHRSP heart by the administration of nifedipine, and the result proves that nifedipine may act as a ligand of PPARγ in SHRSP.

It has been reported that PPARα as well as PPARγ may contribute to reduce the progression of atherosclerosis; however, our results showed little involvement of PPARα in the properties of nifedipine in the SHRSP heart. Our studies also showed little effect of nifedipine on RXR expression, which may also have antiatherogenic effects, suggesting that nifedipine may selectively increase PPARγ in the SHRSP heart, although the precise mechanisms of the nifedipine-induced increase in PPARγ expression and its activity need to be clarified.

It has been reported that antioxidants reduce oxidative stress, improve vascular function and structure, and prevent the progression of hypertension in SHRSP by altering the activation of NAD(P)H oxidase and SOD. We previously reported that another calcium antagonist, amlodipine, reduces ROS and inhibits SMC phenotypic change, a hallmark of vascular dysfunction in hypertension, in SHRSP. The present study showing the marked effects of sub-depressor-dose nifedipine on the phenotype of vascular SMCs, and vascular remodeling suggests that oxida-
Hypertensive stress is one of the most important factors for vascular remodeling, indicating that these processes are redox-sensitive in SHRSP\textsuperscript{28}. NAD(P)H oxidase is a major source of ·O\textsubscript{2}\textsuperscript{−} production, and overexpression of these cytosolic protein might lead to vascular hypertrophy and remodeling in hypertension\textsuperscript{38,39}; however, in the present study, expressions of the membrane-bound subunit p22\textsubscript{phox} and cytosolic component p47\textsubscript{phox} of NAD(P)H oxidase were unaffected by nifedipine, and other ROS-related enzymes did not differ among the three SHRSP groups, suggesting that nifedipine might reduce oxidative stress by selective restoration of Cu/ZnSOD. It has also been reported that PPAR\textgamma ligands inhibit vascular SMC proliferation in hypertension\textsuperscript{40}. Taken together with the present results, it is suggested that in the SHRSP heart, nifedipine might work through the PPAR\textgamma pathway to inhibit vascular remodeling and dysfunction.

Hypertension is characterized by impaired endothelium-dependent vasodilation, since oxidative stress causes a reduction in NO availability\textsuperscript{2}. The potential mechanism by which calcium channel antagonists exert their beneficial activity on endothelial dysfunction may not involve direct calcium antagonism at the level of the endothelium since endothelial cells do not express voltage-sensitive calcium channels to a significant extent. Rather, calcium channel antagonists may exert antioxidant effects that enhance basal NO formation through increasing the expression of endothelial NO synthase, resulting in improved endothelial function\textsuperscript{41}. It has been reported that nifedipine increases NO bioactivity by the activation of SOD\textsuperscript{16} and the functional importance of Cu/ZnSOD in the vasculature\textsuperscript{4}. In the present study, eNOS expression was unaffected by nifedipine in the SHRSP heart with or without GW9662. It has also been reported that PPAR\textgamma agonists stimulate NO release through a mechanism related to NO production\textsuperscript{42} but unrelated to eNOS expression\textsuperscript{43}, and decrease NAD(P)H oxidase-dependent ·O\textsubscript{2}\textsuperscript{−} production\textsuperscript{41}. Although in the present study, circulating concentrations of NO metabolites were not measured, they may follow a similar pattern to the relaxant responses in SHRSP groups. Our results suggest that nifedipine may improve vascular function through an endothelium-independent PPAR\textgamma pathway and reduce oxidative stress via Cu/ZnSOD as previously reported\textsuperscript{43}.

![Fig. 5. Nifedipine induces dose-dependent upregulation of PPAR\textgamma expression in cultured vascular SMCs](image1)

![Fig. 6. Concentration-response curves of relaxation due to ACh in superior mesenteric arterial strips precontracted with NE (10\textsuperscript{−5} mol/L) in WKY and SHRSP](image2)
Conclusion

Our study demonstrates the novel concept that nifedipine may directly and selectively activate PPARγ, and also inhibit intramyocardial arterial remodeling and perivascular fibrosis, improve vascular function beyond blood-pressure lowering and exert antiatherogenic antioxidative action by selectively restoring Cu/ZnSOD in SHRSP.

Acknowledgments

Ms. Rie Ishihara and Yoko Okamoto are acknowledged for their excellent technical assistance.

Sources of Funding

This study was supported in part by Grant-in-Aid for Scientific Research (16590691, 18590776, and 21590549) of Japan.

References

11) Inoue I, Goto S, Matsunaga T, Nakajima T, Awata T, Hohki S, Komoda T, Katayama S: The ligands/activators for peroxisome proliferator-activated receptor α (PPARα) and PPARγ increase Cu²⁺, Zn²⁺-superoxide dismutase and decrease p22phox message expressions in primary endothelial cells. Metabolism, 2001; 50: 3-11
20) Kawahara S, Umemoto S, Tanaka M, Umeji K, Matsuda S, Fuchihata H, Itoh H, Yokozaki H, Yokoyama M: This study was supported in part by Grant-in-Aid for Scientific Research (16590691, 18590776, and 21590549) of Japan.
21) Inoue I, Goto S, Matsunaga T, Nakajima T, Awata T, Hohki S, Komoda T, Katayama S: The ligands/activators for peroxisome proliferator-activated receptor α (PPARα) and PPARγ increase Cu²⁺, Zn²⁺-superoxide dismutase and decrease p22phox message expressions in primary endothelial cells. Metabolism, 2001; 50: 3-11
shev N, Alexander RW: Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to hindlimb ischemia. Circulation, 2005; 111: 2347-2355
26) Zhao R, Shen GX: Functional modulation of antioxidant enzymes in vascular endothelial cells by glycated LDL. Atherosclerosis, 2005; 179: 277-284
36) Chen X, Touyz RM, Park JB, Schiffrin EL: Antioxidant effects of vitamins C and E are associated with altered activation of vascular NADPH oxidase and superoxide dismutase in stroke-prone SHR. Hypertension, 2001; 38: 606-611
37) Fujii K, Umemoto S, Fujiy A, Yonezawa T, Sakumura T, Matsuzaki M: Angiotensin II type 1 receptor antagonist downregulates nonmuscle myosin heavy chains in spontaneously hypertensive rat aorta. Hypertension, 1999; 33: 975-980