

Inhibition of experimental abdominal aortic aneurysm progression by nifedipine

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Abstract. Agents to inhibit the renin-angiotensin system have been reported to suppress the progression of abdominal aortic aneurysm (AAA). However, the effects of calcium channel blockers (CCBs) are still unclear in terms of the inhibition of the progression of AAA. Recently, several effects of CCBs beyond those associated with blood pressure lowering have attracted much interest. In this study, we examined the effects of nifedipine on AAA progression. AAA was induced in rats by transient aortic perfusion with elastase. Then, nifedipine (10 mg/kg/day) and saline (control) were administered to rats by osmotic mini-pump. At 2 and 4 weeks, the size of the AAA, blood pressure and heart rate were measured. Then, to further explore the mechanisms of the progression of AAA, we used human vascular smooth muscle cells (VSMCs). Especially, we focused on NF- κ B and matrix metalloproteinase-9 (MMP-9). Treatment with nifedipine resulted in a significant inhibition of the progression of AAA such as aneurysmal dilation at 14 and 28 days compared to the control (week 2: control, 2.98 ± 0.71 mm; nifedipine, 2.37 ± 0.64 mm; $p < 0.05$ and week 4: control, 3.28 ± 0.98 mm; nifedipine, 2.41 ± 0.17 mm; $p < 0.05$). Neither nifedipine nor saline changed blood pressure and heart rate, significantly. Nifedipine ($1 \mu\text{M}$) significantly suppressed angiotensin II-induced (10^{-6} M) NF- κ B activity in VSMCs by reporter assay ($p < 0.01$). Furthermore, nifedipine ($1 \mu\text{M}$) inhibited MMP-9 protein expression and activity. Saline did not show such inhibitory effects. Taken together, these results indicated that nifedipine inhibits the progression of experimental AAA possibly through suppression of NF- κ B and MMP-9 activity, leading to protective effects against AAA beyond those associated with blood pressure lowering.

Introduction

Abdominal aortic aneurysm (AAA) develops in 4-8% of men and 0.5-1.5% of women older than 50 years of age (1,2). The most important complication is rupture leading to sudden death, which occurs in up to a third of patients left untreated (3). The main pathogenesis of AAA may be based on atherosclerosis. Epidemiological research has revealed that risk factors for AAA are aging, male sex, hyperlipidemia and smoking (4,5). It has been reported that patients with AAA are more likely to have hypertension than patients without AAA (6). However, it is still unclear how hypertension is associated with AAA progression.

The basic phenomena in the pathogenesis of AAA are degradation of extracellular matrix components and loss of structural integrity of the aortic wall (7,8). Moreover, AAA typically involves tissue inflammation as shown by the presence of inflammatory cells, which are considered to participate in the immunopathogenesis of AAA leading to destruction of the aortic matrix (9-11). Investigations have emphasized disease mechanisms involving chronic aortic wall inflammation and the progressive degradation of fibrillar matrix proteins (12). Matrix metalloproteinases (MMPs) play important roles in such mechanisms of AAA, and pathological vascular remodeling is considered to be mediated by MMPs secreted by invasive macrophages, migrating vascular smooth muscle cells (VSMCs), and endothelial cells (ECs) (13-15). The expression of MMP-2, 3, 9 and 12 was significantly increased in harvested human aneurysms (16,17). These findings suggest that MMPs are strongly associated with disease activity. Some researchers have reported inhibitory effects of MMP inhibitors on the progression of experimental AAA (18-20). Moreover, we demonstrated up-regulation of the transcription factor nuclear factor κ B (NF- κ B), which regulates inflammation (21). In turn, NF- κ B regulates the transcription of MMP-1, 2, 3 and 9 (22-24).

Anti-hypertensive drugs, especially angiotensin-converting enzyme (ACE) inhibitors, might slow the progressive course of AAA based on studies in animals (25,26). However, unfortunately, such favorable effects for calcium channel blockers (CCBs) have not been reported, and their effects are still unclear. In this study we investigated the usefulness of one of the CCBs, nifedipine, in an experimental model of AAA.

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Materials and methods

Materials. Nifedipine was donated by Bayer Pharmaceutical Co. (Osaka, Japan). Angiotensin (Ang) II was purchased from Sigma Chemical Co. (St. Louis, MO).

Procedure involved in the AAA model. Male Wistar rats (400–500 g) (Charles River Breeding Laboratories, Osaka, Japan) were anesthetized and underwent laparotomy (19). Briefly, the abdominal aorta was isolated from the level of the left renal vein to the bifurcation. The right femoral artery was exposed, and a PE-10 polyethylene tube (Baxter Healthcare Corp., Deerfield, IL) was introduced through the femoral artery to the distal aorta. The aorta was clamped above the level of the tip of the PE tube and ligated with a silk suture near the aortic bifurcation, followed by perfusion with 0.2 ml saline containing 50 U type I porcine pancreatic elastase (Sigma Chemical Co.). Aortic perfusion with 2 ml saline containing 25 U elastase was performed for 30 min at 100 mmHg. One week after the operation, nifedipine (10 mg/kg/day) and saline were administered to rats by an Alzet osmotic mini pump for 4 weeks (Durect Corp., Cupertino, CA). This experiment was performed under the supervision of the Animal Committee in accordance with the Guidelines on Animal Experiments of Osaka University Graduate School of Medicine.

Ultrasonography, and blood pressure and heart rate measurement. Ultrasonography was used to demonstrate dilation of the AAA. A cardiovascular ultrasound system (Power Vision 6000, Toshiba, Tokyo, Japan) and a linear transducer (15 MHz) were used to image the abdominal aorta noninvasively in anesthetized rats. Rats were scanned transversely to obtain images for measurement of the luminal diameter and the area of the lumen of the aneurysm at the segment with maximum diameter. The aortic size was measured before and after laparotomy once per week up to 4 weeks after the operation.

Blood pressure was measured in pre-warmed rats by the tail-cuff method (BP-98A; Softron Co., Tokyo, Japan) as previously reported (27).

Cell culture. Human VSMCs (passage 3) were obtained from Cloneteck Corp. (Walkersville, MD) and cultured in 10% Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum. Cells were incubated at 37°C in a humidified atmosphere of 95% air–5% CO₂ with medium changes every two days.

NF- κ B reporter assay. In VSMCs NF- κ B activity was evaluated using firefly luciferase reporter plasmid (pNF- κ B-Luc) (Stratagene, La Jolla, CA), which contains the luciferase gene driven by a promoter containing a TATA element and 3 copies of the κ B cis-acting element. Lipofectamine (Invitrogen, Carlsbad, CA) was used to transfer pNF- κ B-Luc into VSMCs. To induce the up-regulation of NF- κ B activity, Ang II (10⁻⁶ M) was used in this study. Cells were collected 48 h after transfer, and cell extracts were prepared using the reporter lysis buffer in the Luciferase Assay System (Promega, Madison, WI). Protein content was

determined using bovine serum albumin (BSA) as a standard. Luciferase values were measured by the Luciferase Assay System. The luciferase values were normalized by the protein content.

Gel mobility shift assay. Nuclear extract was prepared from VSMCs as previously described (28,29). A double-stranded NF- κ B oligonucleotide (ODN) probe was ³²P-labeled with a 3'-end labeling kit (Clontech, Palo Alto, CA) as described previously (28,29). After end-labeling, the ³²P-labeled ODN probe was purified by Nick column (Amersham Pharmacia Biotech, Buckinghamshire, UK). Then, 10 ml of a mixture of ³²P-labeled ODN probe (0.5–1 ng, 20,000 cpm) and 1 mg of polydeoxyinosinic-polydeoxycytidic acid (Sigma Chemical Co.) was incubated with 10 μ g of nuclear extracts from VSMCs for 30 min at room temperature prior to loading onto a 5% polyacrylamide gel. The gels were subjected to electrophoresis, dried, and the labeled DNA was visualized by autoradiography (28,29).

Western blotting. Western blotting was performed for analysis of MMP-9. VSMCs were seeded onto 10-cm dishes (Corning Japan, Tokyo, Japan). Cells were collected after 48 h of stimulation with Ang II and were stored at -80°C until extraction. After treatment, total protein was extracted with RIPA buffer (50 mM Tris-Cl, 0.15 M NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton-X and 10 mM NaF). Samples containing 10, 20 and 40 μ g protein were run on 12.5% sodium dodecylsulfate polyacrylamide gels. Proteins were separated by SDS/PAGE, transferred to a nitrocellulose membrane (Hybond ECL™) (Amersham Pharmacia Biotech), and incubated with a monoclonal antibody to MMP-9 (R&D Systems, Minneapolis, MN) at 4°C overnight. Antibodies were diluted with 4% skimmed milk and 0.1% Tween-20 in PBS. The membrane was then washed and incubated with a 1:2000 dilution of mouse or rabbit IgG horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech). The bound antibody was detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) and Hyperfilm™-MP (Amersham Pharmacia Biotech).

SDS-PAGE zymography. Protein extracts (30 μ g) were mixed with SDS sample buffer (Invitrogen), and each sample was applied to 10% polyacrylamide gel containing 0.1% gelatin under nonreducing conditions. After electrophoresis, the gel was renatured in renaturing buffer (Invitrogen) for 30 min and equilibrated in developing buffer (Invitrogen) for 30 min at room temperature. After equilibration, fresh developing buffer was added, and the gel was incubated overnight at 37°C. The gel was stained with 0.5% Coomassie blue for 30 min and destained with destaining solution containing 10% acetic acid and 40% methanol in distilled water. Areas of protease activity appeared as unstained bands against a blue background.

Statistical analysis. All values were expressed as the mean \pm SEM. Analysis of variance with the subsequent use of the Bonferroni/Dunnet's test was employed to determine the significance of differences in multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

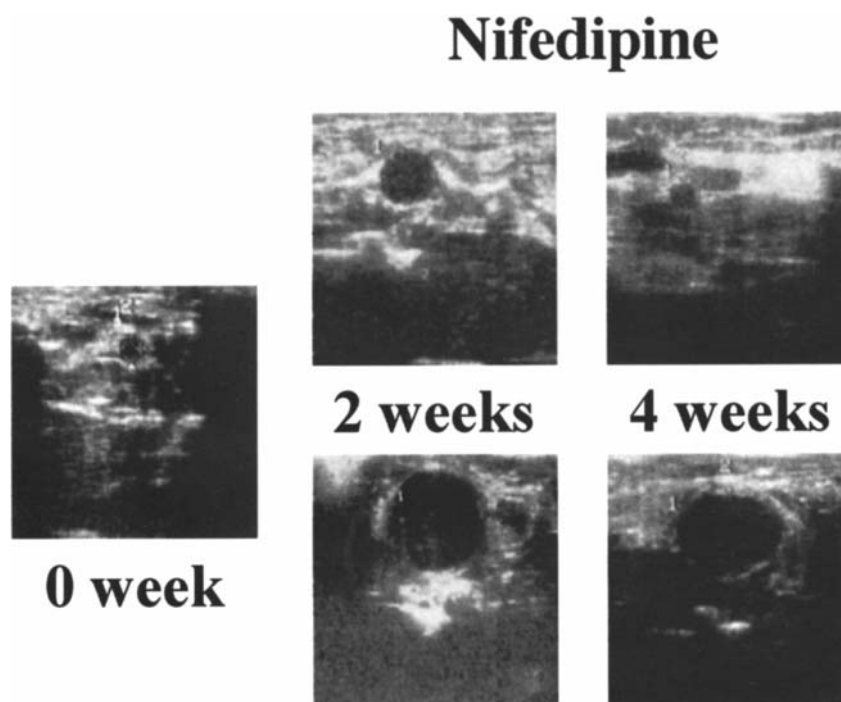


Figure 1. Effects of nifedipine on the size of AAA as assessed by ultrasound. Representative ultrasound images of aortic dilations are shown at each time point (2 and 4 weeks). Week 0 indicates the time point before administration of nifedipine and saline (control); one week after induction of AAA.

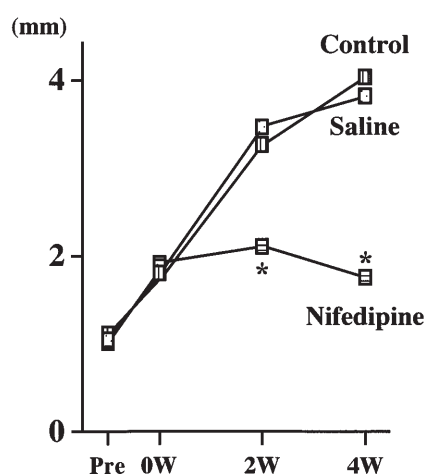


Figure 2. Time course of aortic size. Measurements were performed during ultrasound analysis. Week 0 indicates the time point before administration of nifedipine and saline (control); one week after induction of AAA. n=6/group. Values are the means \pm SEM. * $p < 0.05$ vs. saline and control, respectively.

Results

Prevention of aneurismal dilation by nifedipine. First, we examined the inhibitory effects of nifedipine on aortic aneurismal dilation. As shown in Fig. 1, ultrasound analysis demonstrated that treatment with nifedipine prevented the progression of aortic dilation after elastase infusion. Even at 4 weeks the progression of AAA was still inhibited. To determine the inhibitory effects of nifedipine quantitatively, we measured the diameter of AAA by ultrasound. The data were consistent with the ultrasound analysis as shown in

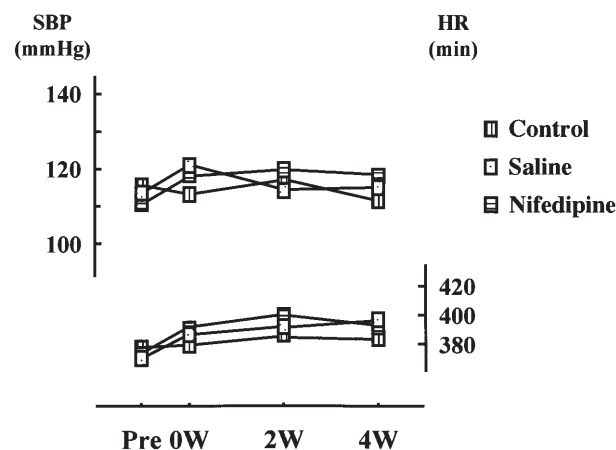


Figure 3. Changes in blood pressure and heart rate. Week 0 indicates the time point before administration of nifedipine and saline (control); one week after induction of AAA. n=6/group. Values are the means \pm SEM.

Fig. 2. Nifedipine significantly suppressed the progression of aortic dilation compared to the control groups ($p < 0.05$).

We used an anti-hypertensive agent, nifedipine, at a dose that did not affect blood pressure. We selected the dose based on the results of our preliminary experiments (data not shown). In this study we also measured blood pressure and heart rate. As shown in Fig. 3, blood pressure and heart rate did not differ between the nifedipine and the control groups, as we expected.

Molecular mechanism of inhibitory effect of nifedipine. It has been reported that the transcription factor NF- κ B plays an important role in the pathogenesis of AAA (30). Therefore, we examined whether or not nifedipine affected NF- κ B

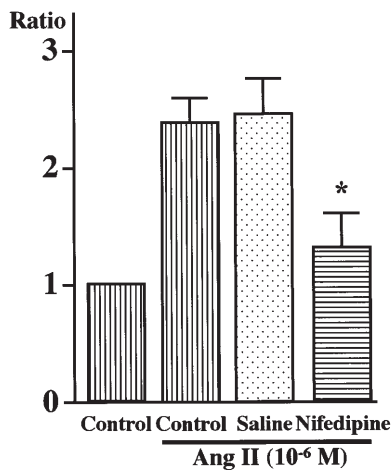


Figure 4. Effects of nifedipine and saline on NF- κ B transcriptional activity in human VSMCs at 48 h. Normalized luciferase activity to renilla activity in control cells was determined as 1.0. Relative firefly luciferase activity are shown. Five sets of experiments were performed. * $p < 0.05$ vs. saline.

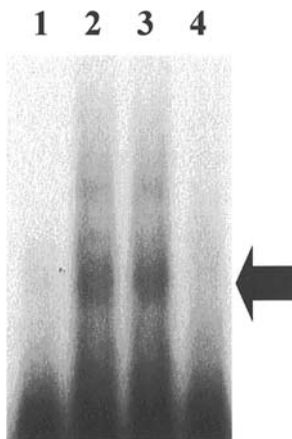


Figure 5. Representative gel mobility shift assay for NF- κ B binding site. Lane 1, unstimulated control; 2, control stimulated with Ang II; 3, saline-treated VSMCs with Ang II (10^{-6} M); and 4, nifedipine-treated VSMCs stimulated with Ang II (10^{-6} M). In each lane 30 μ g of nuclear protein was incubated. Experiments were repeated 4 times.

activity in VSMCs. Compared with the control, nifedipine significantly decreased the expression of luciferase protein stimulated with Ang II (10^{-6} M) ($p < 0.05$) (Fig. 4). This result indicates that nifedipine suppresses the NF- κ B transcriptional activity in VSMCs. To further clarify this inhibitory effect of nifedipine, we performed gel mobility shift assay. This assay also revealed that nifedipine suppressed NF- κ B transcriptional activity in VSMCs (Fig. 5). In contrast, saline, the control, did not decrease NF- κ B activity. These results are consistent with a previous report by Matsumori *et al* (31).

Moreover, researchers have reported inhibitory effects of MMP inhibitors on the progression of experimental AAA, indicating the important role of MMPs under the regulation of NF- κ B. We focused on MMP-9 in this study, because, among MMPs, MMP-9 is reported to be most important in the pathogenesis of the progression of AAA both in humans and animal models. As shown in Fig. 6, nifedipine suppressed MMP-9 protein expression as assessed by Western blot

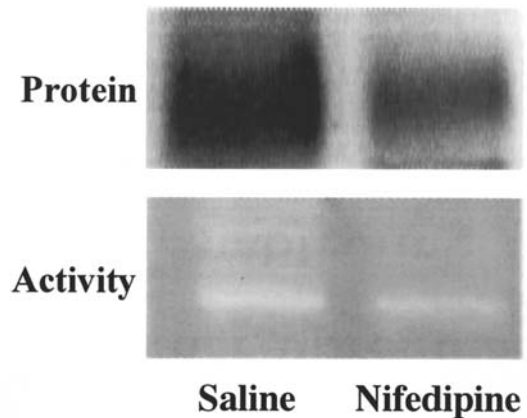


Figure 6. Representative Western blot analysis and zymography for MMP-9. Protein, Western blot analysis; activity, zymography. Experiments were performed 4 times.

analysis. Zymography also showed that nifedipine inhibited the enzyme activity of MMP-9 in VSMCs. However, these inhibitory effects on MMP-9 were not observed in the saline-treated control group.

Discussion

The incidence of AAA is increasing with aged standardized death rates having risen 20-fold in men and 11-fold in women over 30 years (32). The ageing population and improved diagnosis may account for part of this increase, however, a clear rise in the prevalence of AAA has been demonstrated. Although the increase in the rate of AAA in hypertensive patients is greater (33), it remains unclear whether hypertension is one of the risk factors for AAA. Recently, emerging evidence links the renin-angiotensin system to the development of AAA (34). Ang II is strongly up-regulated in human AAA samples (35,36). Based on these reports, hypertension has become recognized as a risk factor for the development and rupture of AAA. However, the control of hypertension is often insufficient for stabilizing the aneurismal wall (37). In randomized trials of patients with AAA, the β -blocker, propranolol, lowered blood pressure, but it did not affect the aneurismal expansion, the need for surgical repair or mortality (38-40). Moreover, no association between β -blocker therapy and rupture has been reported (37). In experimental animal models, ACE inhibitors were substantially more effective at preventing aneurysm growth and rupture than were other anti-hypertensive agents, including CCBs, hydralazine, spironolactone, and angiotensin receptor blockers (ARBs) (25,26,41,42). ARB is a rather new class of drug, so more data based on animal and human studies should be evaluated. Thus, ACE inhibitors might be distinct in affecting the pathophysiology of AAA at present (37).

The mechanisms of AAA involve vascular inflammation related to several inflammatory cytokines, including interleukin (IL)-1, IL-6 and TNF- α (43,44). Human aneurismal tissues are characterized by chronic aortic wall inflammation, and the progressive degradation of fibrillar matrix proteins (12) and MMPs, including MMP-2 and MMP-9, are thought to contribute to aneurysm development (45). Also the relation of inflammation to AAA is suggested by a report



SPANDIDOS PUBLICATIONS stating that monocyte chemoattractant protein-1 and CCR-2, play an important role in Ang II-induced acceleration of the atherosclerotic process and Ang II-induced AAA formation (46). Interestingly, a strong relationship between vascular inflammation and the progression of AAA was reported based on clinical findings (47,48). Thus, hypertension might accelerate the development of AAA through inflammatory changes, since a large number of studies have reported a significant increase in the expression or activation of MMPs, intracellular adhesion molecule (ICAM) and NF- κ B (49,50). The usefulness of ACE inhibitors in preventing the progression and rupture of AAA may be due to the fact that they have a strong effect beyond that associated with blood pressure lowering, such as an anti-inflammatory effect.

In this study we focused on the CCB, nifedipine, as one report revealed that it has a strong inhibitory effect on the activation of NF- κ B in human epithelium-like lung carcinoma cells (31). A previous study showed that amlodipine, diltiazem and verapamil stimulate NF- κ B transcriptional activity in VSMCs (51). Accumulating evidence suggests that therapeutically effective doses of CCBs activate calcium-independent signal transduction pathways altering gene expression (52-54). Thus CCBs directly activate the transcription factor NF- κ B in human VSMCs, independently of intracellular calcium levels (51). In the present study, we demonstrated that nifedipine inhibited the activation of NF- κ B in human VSMCs, which is consistent with a previous report (31). The precise explanation for this difference remains to be determined, though it has been suggested that the inhibitory effect of CCBs on NF- κ B is apparently independent of the action on L-type calcium channels (31). Thus nifedipine may possess the unique property of inhibiting NF- κ B independently of its calcium channel blocking activity, which may partly explain its effectiveness in the prevention of the progression of AAA in this study.

NF- κ B was first identified as a regulator of the expression of the κ light-chain gene in murine B lymphocytes (55), and has been substantially found in many different cells. It regulates the expression of several genes involved in immune and inflammatory responses (56). NF- κ B is known to regulate the transcription of MMP-1, -2, -3 and -9. Additionally, it is worth noting that the contribution of the inflammatory process is also important. In this study we observed inhibition of MMP-9 activity and protein expression by nifedipine, leading to the prevention of the progression of AAA. Taken together, these findings indicate that nifedipine exerted an inhibitory effect on the progression of AAA via the inhibition of NF- κ B, resulting in a decrease in MMP-9 activity. Furthermore, in this study, nifedipine did not decrease blood pressure and heart rate. These effects were confirmed to be beyond those associated with blood pressure lowering.

In conclusion, the CCB, nifedipine, shows potential as an anti-hypertensive drug in the treatment of AAA.

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