Novel Gene Silencer Pyrrole-Imidazole Polyamide Targeting Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Attenuates Restenosis of the Artery After Injury

En-Hui Yao, Noboru Fukuda, Takahiro Ueno, Hiroyuki Matsuda, Koichi Matsumoto, Hiroki Nagase, Yoshiaki Matsumoto, Ayako Takasaka, Kazuo Serie, Hiroshi Sugiyama, Tatsuya Sawamura

Abstract—Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a membrane protein that can support the binding, internalization, and proteolytic degradation of oxidized low-density lipoprotein. The LOX-1 expression increases in the neointima after balloon injury. To develop an efficient compound to inhibit LOX-1, we designed and synthesized a novel gene silencer pyrrole-imidazole (PI) polyamide targeting the rat LOX-1 gene promoter (PI polyamide to LOX-1) to the activator protein-1 binding site. We examined the effects of PI polyamide to LOX-1 on the LOX-1 promoter activity, the expression of LOX-1 mRNA and protein, and neointimal hyperplasia of the rat carotid artery after balloon injury. PI polyamide to LOX-1 significantly inhibited the rat LOX-1 promoter activity and decreased the expression of LOX-1 mRNA and protein. After balloon injury of the arteries, PI polyamide to LOX-1 was incubated for 10 minutes. Fluorescein isothiocyanate–labeled PI polyamide was distributed to almost all of the nuclei in the injured artery. PI polyamide to LOX-1 (100 µg) significantly inhibited the neointimal thickening by 58%. PI polyamide preserved the re-endothelialization in the injured artery. PI polyamide significantly inhibited the expression of LOX-1, monocyte chemoattractant protein-1, intercellular adhesion molecule-1, and matrix metalloproteinase-9 mRNAs in the injured artery. The synthetic PI polyamide to LOX-1 decreased the expression of LOX-1 and inhibited neointimal hyperplasia after arterial injury. This novel gene silencer PI polyamide to LOX-1 is, therefore, considered to be a feasible agent for the treatment of in-stent restenosis. (Hypertension. 2008;52:86-92.)

Key Words: basic science ▪ endothelium ▪ gene therapy ▪ cytokines ▪ polyamide ▪ LOX-1 ▪ restenosis

Coronary artery restenosis after angioplasty occurs in ≈30% of all patients.1,2 Despite the widespread use of intracoronary stents, in-stent restenosis remains a major clinical problem, occurring in ≤50% of high-risk patients.3 The development of neointimal hyperplasia after arterial injury contributes to the pathogenesis of restenosis. Several factors are involved in the initiation and progression of neointimal hyperplasia. Coronary arterial diseases are known to be associated with several risks, such as dyslipidemia, hypertension, smoking, and diabetes. A pivotal common factor in these risks is oxidative stress, which also induces restenosis of the coronary artery.4

The oxidized low-density lipoprotein (ox-LDL) is recognized to be a major cause of endothelial dysfunction in atherogenesis.5 Lectin-like ox-LDL receptor-1 (LOX-1), a receptor for ox-LDL, is a membrane protein that is expressed in both the vascular endothelium and vascular-rich organs. LOX-1 can support the binding, internalization, and proteolytic degradation of ox-LDL.6 The LOX-1 expression has been reported to significantly increase in the neointima after balloon injury in various animal models of neointimal hyperplasia, such as rats and rabbits. Hinagata et al7 reported neointimal hyperplasia after balloon injury to be markedly attenuated by treatment with anti–LOX-1 antibody in a rat model. These findings suggest that LOX-1 expressed in the neointima is involved in the pathogenesis of restenosis after arterial injury, and, therefore, LOX-1 may be a potential therapeutic target for the prevention/treatment of neointimal hyperplasia and restenosis after arterial injury.

Pyrrole-imidazole (PI) polyamide is a powerful gene-regulating compound that can inhibit protein, including enhancers or repressors, DNA binding, and interaction by binding to the minor groove of double-helical DNA with high affinity and specificity.8 PI polyamide was first identified from duocarmycin A and distamycin A, which recognize and bind DNA with sequence specificities and are small synthetic molecules com-
posed of the aromatic rings of N-methylpyrrole and N-methylimidazole amino acids. PI polyamides are resistant to nucleases and do not require any particular delivery systems. Various types of sequence-specific DNA-binding PI polyamides have been developed to control gene expression. DNA recognition depends on a code of side-by-side pairing of pyrrole and imidazole in the minor groove. A pairing of imidazole opposite pyrrole targets the G-C bp, and pyrrole-imidazole targets the C-G bp. Pyrrole-pyrrole degenerately targets the T-A bp and A-T bp. We have reported previously that PI polyamide targeted to the transforming growth factor-β1 promoter for progressive renal diseases significantly inhibited the transforming growth factor-β1 promoter activity and the expressions of transforming growth factor-β1 mRNA and protein in mesangial cells. These findings suggest that the synthetic PI polyamides targeting gene promoter may, therefore, be feasible agents for the treatment of such diseases.

In this study, to develop a new agent for the treatment of restenosis after angioplasty, we designed a PI polyamide targeting rat LOX-1 gene promoter (PI polyamide to LOX-1) and examined its effects on LOX-1 expression and neointimal formation after balloon arterial injury in a rat model.

Methods

Synthesis of Polyamide Targeting Rat LOX-1

PI polyamide to LOX-1 was designed to span the boundary of the AP-1 binding site (–63 to –58) of the LOX-1 promoter (Figure 1A and 1B). A mismatch polyamide was used as a negative control; it was designed not to bind transcription binding sites of the promoter (Figure 1C). PI polyamides were synthesized by Gentier Biosystems Inc, according to methods described previously.

Cell Culture

Rat aortic endothelial cells (Cell Applications) were inoculated on the coated plate and cultured in rat endothelial cell growth medium containing heparin, hydrocortisone, human epidermal growth factor, human fibroblast growth factor, dibutyryl cAMP, and FBS (5% vol/vol final concentration) in a CO₂ incubator. After reaching 90% confluence, the endothelial cells were incubated in serum-free medium for 24 hours, and then the medium was exchanged for a new medium at the start of the experiments.

Reverse Transcription and PCR Analysis

The total RNA was isolated and reverse transcribed as described previously. The primers used to amplify monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinase-9 (MMP-9), and adhesion molecule-1 (ICAM-1) are listed in Table S1 (available online at http://hyper.ahajournals.org/). 18S ribosomal RNA was amplified as an internal control. PCR was performed according to the profiles shown in Table S2. PCR was performed in a DNA thermal cycler (GeneAmp PCR System 2700, Applied Biosystems). The quality and concentration of the amplified PCR products were determined using an Agilent 2100 Bioanalyzer (Agilent).

Arterial Injury and Treatment With Polyamide

This study confirms to the standards of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Male Wistar rats (Charles River Breeding Laboratories) weighing 300 to 350 g were used in all of the experiments. The rats were anesthetized by an IP injection of pentobarbital (100 mg/kg of body weight). The left carotid artery was isolated, and a Fogarty 2F embolectomy catheter (Baxter Healthcare) was introduced through the external carotid arteriotomy incision, advanced to the aortic arch, inflated to produce moderate resistance, and then gradually withdrawn 3 times to produce a distending and de-endothelializing injury. The catheter was removed and the external carotid branch ligated. For local delivery, PI polyamide to LOX-1 or mismatch polyamide was diluted to 10 or 100 μg in 50 μL of saline, and then they were injected and maintained in the artery for 10 minutes. After the incubation period, the solution was evacuated, the artery was washed with PBS 3 times, and then the blood flow through the common carotid artery was re-established.

Distribution of Fluorescein-Labeled Polyamide in Injured Artery

To assess the distribution of the polyamide in the carotid artery after the balloon injury, 100 μg of fluorescein isothiocyanate (FITC)-labeled PI polyamide to LOX-1 was infused within the lumen of the artery for 10 minutes. The vessels were harvested 30 minutes, 2 hours and 24 hours later. Frozen specimens were made and then examined by fluorescence microscopy.

Morphometric Analysis of Neointimal Hyperplasia

The effect of polyamide on neointimal formation was measured as described previously. The rats were euthanized by a lethal injection of sodium pentobarbital (IP, 100 mg/kg of body weight) at 21 days after balloon injury and then perfused with saline followed by 10% formalin at physiological pressure. For immunohistochemistry and a morphometric analysis, the arteries were fixed in 100% methanol overnight, and the middle one third of the common carotid artery was then cut into 4 segments and embedded in paraffin. The specimens were cross-sectioned at a thickness of 3 μm and stained with hematoxylin-eosin. The intima/media cross-sectional area ratios were determined using a computerized apparatus and the National Institutes of Health Image software program (version 1.57).

Immunohistochemistry

Paraffin blocks of the segments of the carotid arteries were used for the immunohistochemistry assay. LOX-1 expression was identified.
with goat polyclonal anti–LOX-1 antibody (1:200, Santa Cruz), and incubated with fluorescein-conjugated chicken antigoat antibody (1:500, Invitrogen). After washing with PBS, the sections were incubated with Hoechst 33342 and then viewed by a laser scanning confocal imaging system.

**Statistical Analyses**

The values are reported as the means±SEMs. Student t test was used for unpaired data. Two-way ANOVA was also used. *P*<0.05 was considered to be statistically significant.

**Results**

Rat LOX-1 Promoter Activity

To identify DNA elements for regulation of rat LOX-1 promoter activation, 4 deletion constructs of the rat LOX-1 promoter were generated (Figure S1A). These constructs were transiently transfected into HEK-293 cells treated without or with phorbol 12-myristate 13-acetate (PMA) and measured for luciferase activity. PMA-induced activity was observed at \(-2385\text{Luc}, -1978\text{Luc}, -1323\text{Luc},\) and \(-128\text{Luc}.\) The luciferase activity of these constructs significantly (*P*<0.05) increased with PMA by 2.8- to 3.2-fold in comparison with the basal activity, whereas the promoterless construct pGL3-basic was unresponsive. These results indicate that the sequence from \(-128\) to \(+24\) is important for PMA-induced activation of the LOX-1 promoter. We searched for transcription factor–binding sites using TFSEARCH and found an AP-1 site (5'-TGCATT-3') lying between bp \(-59\) and \(-53\). To further confirm this AP-1 site on the LOX-1 promoter necessary for LOX-1 promoter activity in response to PMA, a 2-bp mutated construct was made in the luciferase reporter plasmid \(-128\text{Luc}.\) The luciferase activity in the HEK-293 cells transfected with these mutants revealed that mutation of the AP-1 site abolished the effect of PMA on LOX-1 promoter activity (Figure S1B). Therefore, this AP-1 site was essential for promoter activation. The PI polyamide to LOX-1 was then designed to interfere with this site.

**Binding of Polyamide to Target DNA**

The binding affinity and specificity of polyamide to target DNA were determined by gel shift assay (Figure S2). PI polyamide to LOX-1 bound the target double-stranded DNA. However, PI polyamide to LOX-1 did not bind to the 2-bp mutated double-stranded DNA. The mismatch polyamide did not bind to the double-stranded DNA.

**Effect of PI Polyamide to LOX-1 on LOX-1 Promoter Activity**

PMA (0.1 μmol/L) markedly increased the luciferase activity in HEK-293 cells transfected with LOX-1 promoter plasmid.

**Effect of PI Polyamide to LOX-1 on the Expressions of LOX-1 mRNA and Protein in Cultured Rat Endothelial Cells**

The expression of LOX-1 mRNA and protein was significantly (*P*<0.05) increased with PMA. PI polyamide to LOX-1 significantly (*P*<0.05) inhibited the amount of LOX-1 mRNA and protein. However, the mismatch polyamide did not affect the amount of LOX-1 mRNA and protein (Figure S3A and S3B).

**Distribution of PI Polyamide to LOX-1 in Injured Artery**

Figure 3 shows the distribution of FITC-labeled PI polyamide to LOX-1 in rat carotid artery after balloon injury. The FITC-labeled PI polyamide was not seen and then was uptaken into the entire wall of the injured artery at 30 minutes after injury. Thereafter, the FITC-labeled PI polyamide remained and strongly localized in the nuclei of midlayer smooth muscle by 24 hours.

**Effect of PI Polyamide to LOX-1 on Neointimal Thickening**

Figure 4 shows the effect of PI polyamide to LOX-1 on neointimal thickening in rat carotid artery at 21 days after balloon injury. Both 10 and 100 μg of PI polyamide to
LOX-1 significantly ($P < 0.05$) reduced neointimal thickening by 31% and 58%, respectively, in comparison with the injury group. The mismatch polyamide did not affect the neointimal formation.

**Effect of PI Polyamide to LOX-1 on Expression of LOX-1 in the Injured Artery**

The expression of LOX-1 mRNA was significantly ($P < 0.05$) higher in the injured artery than that in the noninjured artery. Treatments with PI polyamide to LOX-1 significantly ($P < 0.05$) reduced the expression of LOX-1 mRNA in the artery at 3, 7, and 21 days after balloon injury. The treatments with mismatch polyamide did not affect the expression of LOX-1 mRNA in the artery (Figure 5). Immunofluorescence staining showed that LOX-1 was not detectable in the noninjured artery, whereas LOX-1 was markedly increased in the endothelial layer and midlayer smooth muscle in the injured artery at 21 days after injury. The treatment of PI polyamide to LOX-1 reduced the LOX-1 expression. The mismatch polyamide did not affect the expression of LOX-1 (Figure 6).

**Effect of PI Polyamide to LOX-1 on Re-endothelialization in the Injured Artery**

Immunohistochemistry of endothelial cells with anti–von Willebrand factor antibody in rat carotid artery at 21 days after balloon injury showed that endothelial cells were stained in the intimal surface after balloon injury. The treatment of PI polyamide to LOX-1 obviously enhanced the staining of the endothelial cells in the injured artery (Figure 6).

**Effect of PI Polyamide to LOX-1 on the Expression of MCP-1, ICAM-1, and MMP-9 mRNAs in Injured Artery**

The expression of MCP-1, ICAM-1, and MMP-9 mRNAs significantly ($P < 0.05$) increased in the injured artery 3 days after injury in comparison with the noninjured artery. The treatment of PI polyamide to LOX-1 significantly ($P < 0.05$) reduced the expression of these mRNAs. The mismatch polyamide did not affect the expression of these mRNAs (Figure 7).

**Discussion**

In the present study, we constructed deletion mutants and analyzed the rat LOX-1 promoter activity stimulated by PMA and found a transcription factor AP-1 binding site between bp −59 and −53 in the rat LOX-1 promoter. The site-directed mutation analysis further confirmed this positive regulatory element for the activation of LOX-1 promoter. These findings imply that, to interfere with this AP-1 binding site, PI polyamide to LOX-1 will suppress the LOX-1 gene activation and expression. For gene-specific targeting, the polyamide was then designed to target the sequence immediately adjacent to the binding site for AP-1 on the promoter. Synthetic PI polyamides have been shown to be cell permeable and able to...
inhibit the transcription of specific genes. In our previous study, we also demonstrated that synthetic PI polyamides easily passed into the cells and then entered the nuclei of cells without any vector or delivery reagents to inhibit the expression of the target gene.\(^8,13\) In this study, in a construct of plasmid, the polyamide significantly inhibited the LOX-1 promoter activity stimulated with PMA, thus suggesting that the synthetic PI polyamide to LOX-1 actually interfered with the AP-1 transcription factor-DNA interaction.

The designed PI polyamide to LOX-1 significantly inhibited the expression of LOX-1 mRNA and protein stimulated with PMA in cultured rat aortic endothelial cells, thus suggesting that PI polyamide to LOX-1 has the potential to control LOX-1 gene expression. LOX-1 has been reported to induce apoptosis of endothelial cells, which is associated with the atherosclerosis and restenosis of artery.\(^7,19\) Because the endothelium has the ability to improve arterial injury, the denudation of the endothelium by coronary intervention may, thus, accelerate the occurrence of restenosis. Drug-eluting stents (DESs) have been shown to be effective for preventing in-stent restenosis. The sirolimus-coated DESs can prevent in-stent restenosis by inducing the complete inhibition of vascular smooth muscle cell hyperplasia by its effect on cell cycle arrest.\(^20\) However, complications such as subacute thrombosis or late thrombosis have been reported recently in patients implanted with a sirolimus-coated DES.\(^21\) Sirolimus prevents re-endothelialization of the inner side of the metal stent, which may cause late thrombosis. These complications have led to the development of second-generation DESs that do not induce late thrombosis. In the present study, the rapid regeneration of endothelial cells may appear to contribute to the suppression of intimal hyperplasia after treatment with PI polyamide to LOX-1. The preservation of the endothelium by PI polyamide to LOX-1 is, thus, considered to be very advantageous for DESs to prevent both restenosis and late thrombosis.

In the present study, FITC-labeled PI polyamide to LOX-1 was well distributed into the wall of the carotid artery and strongly bound the cell nucleus without any vectors after injury. Nucleic acid medicines, such as antisense DNA, ribozymes, and decoy, have been developed as gene-silencing agents. Decoys, in particular, inhibit the binding of target transcription factors in a manner similar to polyamides. However, because these agents tend to easily degrade when coming into contact with nucleases, they require drug-delivery systems for sufficient distribution into organs. In contrast, PI polyamides are completely resistant to nucleases and can be delivered into organs without delivery systems.

Figure 6. Effect of PI polyamide targeting rat LOX-1 promoter (PI polyamide to LOX-1) on the expression of LOX-1 protein and the re-endothelialization of rat carotid artery after balloon injury. A 2F embelectomy catheter with balloon was drawn toward the arteriotomy site 3 times. After the injury, arteries were incubated without (Injury) or with 100 μg of PI polyamide to LOX-1 (Polyamide) or mismatch polyamide (Mismatch) for 10 minutes. At 21 days after balloon injury the carotid arteries were removed and stained with anti-LOX-1 antibody (LOX-1) or anti–von Willebrand factor antibody (Endothelial cell), and incubated with fluorescein-conjugated respective secondary antibodies. The nuclei were stained with Hoechst 33342. Scale bar represents 200 μm for LOX-1 and Hoechst or 50 μm for endothelial cell.

Figure 7. Effect of PI polyamide targeting rat LOX-1 promoter (PI polyamide to LOX-1) on the expression of MCP-1, ICAM-1, and MMP-9 mRNAs in rat carotid artery after balloon injury. A 2F embelectomy catheter with balloon was drawn toward the arteriotomy site 3 times. After the injury, arteries were incubated without (Injury) or with 100 μg of PI polyamide to LOX-1 (Polyamide) or a mismatch polyamide (Mismatch) for 10 minutes. At 3 days after balloon injury, total RNA was extracted and mRNA expression was evaluated by RT-PCR assay. The ratio of mRNA to 18S rRNA was evaluated. The data are the means±SEMs (n=4 to 6). *P<0.05 vs injury.
In the present study, the expression of LOX-1 was low in the noninjury artery, whereas the expression of LOX-1 was significantly increased after balloon injury. PI polyamide to LOX-1 effectively inhibited LOX-1 expression in the injured artery and attenuated the neointimal formation of the artery after injury. LOX-1 has been reported to be expressed in atheromatous lesions and is involved in neointimal hyperplasia after vascular injury. Therefore, LOX-1 is a target for the treatment of restenosis, and the polyamide against LOX-1 may be an effective approach to inhibit restenosis.

LOX-1 is a main receptor for ox-LDL. Ox-LDL plays a role in the initiation and progression of atherosclerosis via LOX-1. Other than ox-LDL, LOX-1 binds multiple classes of ligands that are implicated in the pathogenesis of atherosclerosis by the apoptosis of cells and the activation of platelets. A significant number of apoptotic cells have been reported to be present in restenotic lesions after balloon injury, thus implying that several factors presented after injury may interact with and activate LOX-1. The activation of LOX-1 may, therefore, increase superoxide generation, reduce the production of NO, induce MCP-1, and increase leukocyte adhesiveness. Hinagata et al demonstrated that the inhibition of LOX-1 with anti–LOX-1 antibody attenuated oxidative stress in the neointima of the rat injured artery. In addition, antioxidative agents have been reported to inhibit the neointimal hyperplasia in normocholesterolemic rabbits and pigs. In the present study, the expression of MCP-1, ICAM-1, and MMP-9 mRNAs was markedly increased in the injured artery, which was significantly decreased with treatments of PI polyamide to LOX-1, suggesting that increases in these molecules are associated with the induction of LOX-1 in the injured artery. MCP-1 is a potent chemotactic factor of monocytes and is produced by activated vascular smooth muscle cells or other type of cells. Antisense oligodeoxynucleotides to LOX-1 inhibit MCP-1 and monocyte adhesion. The inhibition of MCP-1 results in a significant attenuation of neointimal hyperplasia. MMP-9 is upregulated after angioplasty and involved in regulating the proliferation and migration of vascular smooth muscle cells, which are crucial steps for intimal hyperplasia. Therefore, the designed PI polyamide to LOX-1 may attenuate intimal hyperplasia through cellular adhesion to the injured artery. This might partially explain the beneficial effects of the PI polyamide targeting LOX-1 on the suppression of neointimal hyperplasia.

In conclusion, the synthetic PI polyamide to LOX-1 potentially suppressed the LOX-1 promoter activity. PI polyamide to LOX-1 was delivered in midlayer smooth muscle of an injured artery without delivery reagents and significantly inhibited the intimal hyperplasia with the downregulation of MCP-1, ICAM-1, and MMP-9 and re-endothelialization in the injured artery. PI polyamide to LOX-1 is a potentially effective agent for the treatment of in-stent restenosis and will be a candidate agent for the development of next-generation DES.

**References**


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Online supplement

A Novel Gene Silencer Pyrrole-Imidazole Polyamide Targeting LOX-1 Attenuates Restenosis of the Artery after Injury

En-Hui Yao¹, Noboru Fukuda¹², Takahiro Ueno¹, Hiroyuki Matsuda¹², Koichi Matsumoto¹, Hiroki Nagase²³, Yoshiaki Matsumoto⁴, Ayako Takasaka⁵, Kazuo Serie⁶, Hiroshi Sugiyama⁷, Tatsuya Sawamura⁸

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Expanded Materials and Methods

Construction of Rat LOX-1 Promoter Deletion and Mutation Constructs

The DNA fragment of the 5'-promoter region was amplified by PCR using rat genomic DNA as the template, the upstream primers tagged with the Kpn I restriction site and the downstream primer tagged with the Sac I restriction site. The amplified fragments were digested with Kpn I and Sac I and subcloned between the Kpn I and Sac I sites of pGL3-basic vector (Promega). The authenticity of each was verified by a sequence analysis in both directions. The AP-1 mutant construct was generated using the Gene
Editor Site-directed mutagenesis kit (Promega). The mutation was confirmed by DNA sequencing.

**Transfection and Luciferase Reporter Gene Assay**

A mixture of LOX-1 promoter plasmid (1 µg/well) and phRG-TK vector (0.01 µg/well; Promega) as an internal control was transfected into HEK-293 cells. Twenty-four hours after transfection, the cells were incubated with either polyamide or mismatch polyamide in the presence or absence of 0.1 µmol/L phorbol 12-myristate acetate (PMA) as a stimulator for LOX-1 promoter activity for 24 hours. The luciferase activity was measured with a Dual-luciferase reporter assay system (Promega) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

**Gel Mobility Shift Assay**

Fluorescein-labeled DNA corresponding to bp -69 to -46 of rat LOX-1 promoter including the AP-1 binding site or 2-bp mutated DNA were synthesized for a gel mobility shift assay. One µmol/L DNA was incubated with 50 µmol/L PI polyamide to
LOX-1 for 1 hour at 37°C, and then was separated by electrophoresis on 20% polyacrylamide gel and visualized by a luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

**Real-time PCR**

The total RNA was isolated and reverse-transcribed as described previously. Real-time quantitative PCR was performed with cDNA diluted 4 times, using a TaqMan Universal Master Mix, and an ABI 7500 sequence detector (Applied Biosystems). Assay-on-Demand primers and probes (LOX-1: Rn00591116_m1; and TaqMan Rodent GAPDH control reagents) were purchased from Applied Biosystems. The comparative CT method was used for relative quantification and statistical analysis.

**Western Blot Analysis for Rat LOX-1 Protein**

Rat endothelial cells were disrupted with lysis buffer. The total proteins were extracted, heated at 95°C for 5 minutes, subjected to 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and electroblotted onto PVDF membranes (Amersham
Biosciences, Uppsala, Sweden). The blots were incubated with goat polyclonal anti-LOX-1 antibody (1:200, Santa Cruz Biotechnology, CA), or mouse monoclonal antibody specific for α-tubulin (1:2000, Sigma) as an internal control and then with anti-goat IgG or anti-mouse IgG (1:2000, Bio-Rad Laboratories, Hercules, CA), respectively, as secondary antibodies. The bound antibodies were detected by enhanced chemiluminescence (ECL Kit, Amersham) and exposure to X-ray films.
Table S1. Sequences of PCR primers for MCP-1, ICAM-1, MMP-9 and 18S rRNA, and product sizes

<table>
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<th>Target mRNA</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product size (bp)</th>
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<td></td>
<td>3’</td>
<td>5’- TTCCTTATTGGGGTCAGCAC -3’</td>
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</tr>
<tr>
<td>ICAM-1</td>
<td>5’</td>
<td>5’- AGGTATCCATCCATCCCCACA-3’</td>
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<td></td>
<td>3’</td>
<td>5’- GCCACAGTTCTCAAGCACA -3’</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>5’</td>
<td>5’- CCACCGAGCTATCCACTCAT-3’</td>
<td>159</td>
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<tr>
<td></td>
<td>3’</td>
<td>5’- GTCCGGTTTCAGCATGTTTT-3’</td>
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<tr>
<td>18S rRNA</td>
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<tr>
<td></td>
<td>3’</td>
<td>5’- GCTATTGGAGCTGGAATTCCG-3’</td>
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PCR, polymerase chain reaction; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; MMP-9, matrix metalloproteinase-9.

Table S2. Thermal cycle profiles for PCR

<table>
<thead>
<tr>
<th>Target mRNA</th>
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<th>Primer annealing</th>
<th>Primer extension</th>
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<td>60°C, 45 sec</td>
<td>72°C, 45 sec</td>
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<tr>
<td>ICAM-1</td>
<td>94°C, 30 sec</td>
<td>60°C, 30 sec</td>
<td>72°C, 30 sec</td>
<td>30</td>
</tr>
<tr>
<td>MMP-9</td>
<td>94°C, 30 sec</td>
<td>60°C, 30 sec</td>
<td>72°C, 30 sec</td>
<td>30</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>94°C, 30 sec</td>
<td>55°C, 30 sec</td>
<td>72°C, 30 sec</td>
<td>20</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; MMP-9, matrix metalloproteinase-9.
A, An analysis of the LOX-1 promoter activity. Deletion constructs of rat LOX-1 promoter were used in the transient transfection experiments and are drawn on the left. HEK-293 cells were transfected with recombinant LOX-1 promoter plasmids, and were stimulated without or with PMA (1 μmol/L) for 24 hours. B, Mutation of the AP-1 site abolished effect of the PMA on LOX-1 promoter activity. The AP-1 mutated LOX-1 promoter construct was used in transient transfection experiments to determine the role
of AP-1 in mediating the PMA-enhanced LOX-1 promoter activity by a luciferase assay.

The data are the mean ± SEM (n = 8). * P<0.05 vs. treatment without PMA.

**Figure S2.**

Gel mobility shift assay. Fluorescein-labeled DNA corresponding to -69 to -46 of rat LOX-1 promoter including the AP-1 binding site or 2-bp mutated DNA was synthesized. One microgram of the DNA was incubated with 50 µmol/L polyamide for 1 hour at 37°C, thereafter it was separated by electrophoresis on 20% polyacrylamide gel. Lane 1, single-stranded DNA; lane 2, double-stranded DNA; lane 3, double-stranded DNA with polyamide targeting LOX-1; lane 4, 2-bp mutated DNA with polyamide; lane 5, double-stranded DNA with mismatch polyamide.
Figure S3.

A

![Bar graph showing mRNA expression (fold) for Control, PMA, Mismatch, Polyamide 0.1, and Polyamide 1.0 (µM).]

B

![Western blot analysis for LOX-1 and α-Tubulin.](image)

![Bar graph showing LOX-1 protein (% control) for Control, PMA, Mismatch, Polyamide 0.1, and Polyamide 1.0 (µM).]
A, The effect of PI polyamide to LOX-1 on the expression of LOX-1 mRNA in cultured rat aortic endothelial cells. Rat aortic endothelial cells were incubated with PI polyamide to LOX-1 or mismatch polyamide in the presence or absence of 0.1 µmol/L PMA for 6 hours. Total RNA was extracted and LOX-1 mRNA were evaluated by a real-time PCR assay. B, A Western blot analysis of the expression of LOX-1 protein in cultured rat endothelial cells. The endothelial cells were incubated with either PI polyamide to LOX-1 or mismatch polyamide in the presence or absence of 0.1 µmol/L PMA for 12 hours. The data are the mean ± SEM (n = 4). * P<0.05 vs. incubation with PMA.