Atorvastatin Prevents Endothelial Dysfunction in Mesenteric Arteries From Spontaneously Hypertensive Rats
Role of Cyclooxygenase 2–Derived Contracting Prostanoids

Agostino Virdis, Rocchina Colucci, Daniele Versari, Narcisa Ghisu, Matteo Fornai, Luca Antonioli, Emiliano Duranti, Elena Daghini, Chiara Giannarelli, Corrado Blandizzi, Stefano Taddei, Mario Del Tacca

Abstract—We investigated the effect of atorvastatin on cyclooxygenase (COX) contribution to endothelial dysfunction in spontaneously hypertensive rat (SHR) mesenteric resistance arteries. Atorvastatin (10 mg/kg per day, oral gavage) or its vehicle was administered for 2 weeks to male SHR or Wistar-Kyoto rats. Endothelial function of mesenteric arteries was assessed by pressurized myograph. In Wistar-Kyoto rats, relaxation to acetylcholine was inhibited by N\textsuperscript{G}-nitro-L-arginine methyl ester and unaffected by SC-560 (COX-1 inhibitor), DuP-697 (COX-2 inhibitor), or ascorbic acid. In SHRs, the response to acetylcholine was attenuated, less sensitive to N\textsuperscript{G}-nitro-L-arginine methyl ester, unaffected by SC-560, and enhanced by DuP-697 or SQ-29548 (thromboxane-prostanoid receptor antagonist) to a similar extent. Endothelium-dependent relaxation was normalized by ascorbic acid or apocynin (NADPH oxidase inhibitor), which also restored the inhibition by N\textsuperscript{G}-nitro-L-arginine methyl ester. In atorvastatin-treated SHRs, relaxation to acetylcholine was normalized, fully sensitive to N\textsuperscript{G}-nitro-L-arginine methyl ester, and not affected by SC-560, DuP-697, SQ 29548, or antioxidants. Dihydroethidium assay showed an increased intravascular superoxide generation in SHRs, which was abrogated by atorvastatin. RT-PCR revealed a COX-2 induction in SHR arteries, which was downregulated by atorvastatin. The release of prostacyclin and 8-isoprostane was higher from SHR than Wistar-Kyoto mesenteric vessels. COX-2 inhibition and apocynin decreased 8-isoprostane without affecting prostacyclin levels. Atorvastatin increased phosphorylated extracellular signal–regulated kinase 1/2, pAkt, peNOS\textsuperscript{1177}, and inducible NO synthase levels in SHR mesenteric vessels and decreased 8-isoprostane release. In conclusion, COX-2–derived 8-isoprostane contributes to endothelial dysfunction in SHR mesenteric arteries. Atorvastatin restores NO availability by increasing phosphorylated extracellular signal–regulated kinase 1/2, pAkt, peNOS\textsuperscript{1177}, and inducible NO synthase levels and by abrogating vascular NADPH oxidase–driven superoxide production, which also results in a downregulation of COX-2–dependent 8-isoprostane generation. (Hypertension. 2009;53:00-00.)

Key Words: cell signaling ■ endothelium ■ microcirculation ■ oxidant stress ■ NO

Genetic hypertension is characterized by vascular endothelial dysfunction resulting mainly from increased generation of reactive oxygen species (ROS), which, in turn, cause NO breakdown.\textsuperscript{1,2} It is accepted that such endothelial dysfunction is implicated in the pathogenesis of atherosclerosis, leading to an increased risk of cardiovascular events.\textsuperscript{3} There is evidence indicating that cyclooxygenase (COX)-derived prostanoids are also involved in endothelial dysfunction. In particular, studies in spontaneously hypertensive rats (SHR) have shown that COX-2 can produce contracting prostanoids, which act on thromboxane-prostanoid (TP) receptors to maintain a pathological modulation of vascular responses.\textsuperscript{4–7} The ROS excess is hypothesized as 1 possible mechanism for the increased vasmotor COX-2 activity in the setting of hypertension.\textsuperscript{4,7} Inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (statins) exert a lipid-independent amelioration of endothelial dysfunction,\textsuperscript{8,9} mainly by an attenuation of NADPH oxidase–mediated superoxide generation.\textsuperscript{9} An interaction between statins and the COX pathway has been described previously. In aorta from aged rats, simvastatin inhibited the generation of COX-2–derived contracting prostanoids.\textsuperscript{10} In addition, atorvastatin reduced COX-2 expression in a rabbit model of atherosclerosis.\textsuperscript{11} However, the impact of statins on the role of COX-2–derived prostanoids in endothelial dysfunction of peripheral resistance vessels in hypertension is presently unknown. Another statin-activated signaling cascade, leading to the restoration of endothelial function, consists of activation of the phosphatidylinositol 3-kinase/Akt/endothelial NO synthase (NOS; eNOS) pathway,\textsuperscript{8,9} an effect that can be
partly mediated by activation of the extracellular signal-regulated kinase (ERK)-1/2.\textsuperscript{12} The involvement of such pathways in peripheral resistance vessels remains undetermined.

The SHR is a model of genetic hypertension characterized by vascular endothelial dysfunction attributable to increased NADPH oxidase–driven ROS generation, and it is associated with vascular expression and activity of COX-2 in the absence of lipid disorders. Therefore, in the present study we used the SHR model to investigate the effects of atorvastatin on endothelial dysfunction, as well as the involvement of ROS, COX-2, and prostanoids resulting from its activity in mesenteric resistance arteries. The activation of phosphati-
dylinositol 3-kinase/Akt/eNOS and ERK-1/2 signaling path-
ways as possible mechanisms recruited by atorvastatin in this vascular district was also assessed.

Methods

Animals

Experiments were performed in accordance with the European Union Council Directive 86-609, recognized by the Italian government. Male SHRs and Wistar-Kyoto rats (WKYs; Charles River Italy, 12 weeks old) were allocated to receive atorvastatin (Pfizer; 10 mg/kg per day by oral gavage) or vehicle for 2 weeks (n = 6 animals per group). The dose of atorvastatin was selected according to preliminary dose-titration experiments (from 1 to 20 mg/kg per day) to obtain a maximal functional effect without any hemodynamic interference (data not shown). Systolic blood pressure was measured by the tail-cuff method. The average of 3 pressure recordings was obtained. At the end of treatments, rats were euthanized under anesthesia with chloral hydrate.

Preparation of Small Mesenteric Arteries and Functional Experiments

A third-order branch of the mesenteric arterial tree was dissected and mounted on 2-glass microcannulae in a pressurized myograph, as described previously.\textsuperscript{13} Vessels were equilibrated for 60 minutes under constant intraluminal pressure (45 mm Hg) in warmed (37°C) and bubbled (95% air and 5% CO\textsubscript{2}) Krebs’ solution, as detailed elsewhere.\textsuperscript{13}

Endothelium-dependent and -independent relaxations were assessed by measuring the dilatory responses of mesenteric arteries to cumulative concentrations of acetylcholine (ACh; 0.001 to 100 \textmu mol/L) and sodium nitroprusside (0.01 to 100 \textmu mol/L), respectively, in vessels precontracted with norepinephrine (NE; 10 \textmu mol/L). To avoid the possibility that NE might induce different contractility in vessels from controls, SHRs, or atorvastatin-treated animals and to establish which NE concentration was able to elic-
similar contractions among the groups, we performed a concen-
tration-response analysis of NE effects (from 1 nmol/L to 100 \textmu mol/L) in preliminary experiments (data not shown). After the titration study, the NE concentration of 10 \textmu mol/L, which induced similar contracting responses in all of the groups, was selected (Table).

### Influence of COX-1 and COX-2 Activity, TP Receptors, and ROS Production on Endothelium-Dependent Relaxation

The participation of COX-1 and COX-2 isoenzymes in the modula-
tion of endothelial function was assessed by constructing concentration-response curves to ACh after 30-minute preincubation with SC-560 (1 \textmu mol/L; COX-1 inhibitor)\textsuperscript{15} or Dup-697 (1 \textmu mol/L; COX-2 inhibitor).\textsuperscript{15} To ascertain the contribution of TP receptors to endothelial dysfunction, concentration-response curves to ACh were also repeated after 30-minute preincubation with SQ-29548 (1 \textmu mol/L; TP receptor antagonist).\textsuperscript{16} The participation of thromboxane (TX) A\textsubscript{2} to vascular reactivity was assessed by testing ACh in the presence of ozagrel, a TX synthase inhibitor (1 \textmu mol/L).\textsuperscript{17} To assess the influence of ROS on endothelial function, the responses to ACh were determined after 30-minute incubation with ascorbic acid (100 \textmu mol/L).

### Influence of NO Availability and NADPH Oxidase Inhibition on Endothelium-Dependent Relaxation

To evaluate NO availability, concentration-response curves to ACh were determined before and after 30-minute preincubation with the NOS inhibitor N\textsuperscript{3}-nitro-l-arginine methyl ester (l-NAME; 100 \textmu mol/L). To assess the role of NADPH oxidase, ACh was assayed after 30-minute incubation with the NADPH oxidase inhib-
itor apocynin (10 \textmu mol/L).\textsuperscript{18} Moreover, to examine whether NADPH oxidase was involved in decreasing NO availability, ACh was tested during incubation with l-NAME plus apocynin. In additional experiments on SHR vessels (n = 6), the possible influence of COX-2 on NO availability was assessed by testing ACh under the presence of l-NAME plus Dup-697.

### Detection of Vascular Superoxide Anion

The production of superoxide anion from 30-\textmu mol frozen mesenteric vessel sections was evaluated by means of the fluorescent dye dihydroethidium (DHE), as described previously.\textsuperscript{19,20} Serial sections were equilibrated under identical conditions for 30 minutes at 37°C in Krebs-HEPES buffer. Fresh buffer, containing 2 \textmu mol/L of DHE, was applied onto each section, coverslipped, incubated for 30 minutes, and evaluated under fluorescence microscopy. In the presence of superoxide, DHE is oxidized, and it intercalates in DNA, thus staining the nucleus with red fluorescence (excitation at 488 nm and emission 610 nm). The percentage of red-stained arterial wall

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<td>Parameter</td>
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<td>MDA, \textmu mol/L</td>
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<td>Lumen diameter precontracted with NE 10 \textmu mol/L, ( \mu \text{m} ) (%)</td>
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SBP indicates systolic blood pressure; MDA, malondialdehyde; ATO, atorvastatin.

*P < 0.001 vs respective control.
†P < 0.001 vs other groups.
‡P < 0.05 vs respective control.
area was evaluated by imaging computer software (McBiophotonics Image J, National Institutes of Health).

RNA Extraction and RT-PCR
Expression of COX-2 mRNA was assessed by RT-PCR, as described previously.13 Total RNA was isolated from mesenteric vessels by TRizol (Life Technologies, Inc) and chloroform. Total RNA (1 µg) served as a template for cDNA synthesis by Moloney murine leukemia virus reverse transcriptase. cDNA samples were subjected to PCR by specific primers for COX-2.21 Amplification conditions were as follows: 1 minute at 94°C, 2 minutes at 60°C, and 1 minute at 72°C for 35 cycles. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, visualized by UV light, and analyzed by Kodak Image Station 440. The relative expression of COX-2 mRNA was normalized to that of β-actin.

6-Keto-Prostaglandin F1α and 8-Isoprostane Assay
The concentrations of 6-keto-prostaglandin (PG)F1α and 8-isoprostane were determined in the incubation medium of isolated mesenteric vessels at the end of full concentration-response curves to ACh in the absence or in the presence of 30-minute preincubation with Dup-697 (1 µmol/L) or apocynin (10 µmol/L). The prostanoi concentrations were measured by commercial kits for enzyme immunoassays (Cayman Chemical). For this purpose, the incubation medium was frozen in liquid nitrogen immediately after collection, stored at −70°C until processed, and assayed in accordance with the manufacturer’s instructions.

Western Blot Analysis
Tissue samples were homogenized and centrifuged at 15,000g for 15 minutes (4°C). Protein lysates (15 µg) were separated by electrophoresis and transferred onto nitrocellulose membrane. Blots were blocked with 5% nonfat dried milk for 2 hours and incubated overnight at room temperature with antibodies against rat phosphorylated ERK-1/ERK-2 (Thr202/Tyr204; pERK-1/2), phosphorylated Akt (Ser473; pAkt), phosphorylated eNOS (Ser1177; peNOS; Cell Signaling Technology), or inducible NOS (iNOS; Transduction Laboratories). After washing, blots were incubated with peroxidase-labeled antibodies, respectively. The intensities of pERK-1/2 and peNOS immunoactive bands were normalized to that of β-actin. The intensities of pAkt and peNOS immunoactive bands were normalized to that of total Akt and total eNOS, respectively.

Determination of Plasma Malondialdehyde and Cholesterol Assays
Serum was separated by centrifugation and stored at −70°C. The colorimetric assessment of malondialdehyde levels was performed by commercial kit (Cayman Chemical). Total serum cholesterol was assayed by enzymatic method (Roche).

Drugs and Solutions
Apocynin was purchased from Fluka (Sigma-Aldrich). ACh, ascorbic acid, Nω-nitro-l-arginine methyl ester, SC-560 (5-[4chlorophenyl]-1-[4-metoxyphenyl]-3-trifluoromethylpirazole), and sodium nitroprusside were purchased from Sigma Chemicals. SQ-29548 (7-[3-[2-[{phenylamino}carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-1S[1α,2α(Z), 3α,4α]-5-heptenoic acid) was provided by Cayman Chemical. Ozagrel hydrochloride and Dup-697 (5-bromo-2-[4-fluorophenyl]-3-[methylsulfonyl]-thiophene) were supplied by Tocris Bioscience. Drug solutions were prepared in distilled water, except for SC-560, Dup-697, and SQ-29548 (dimethylsulphoxide). Further dilutions were prepared with distilled water.

Data Analysis
Results are presented as mean±SEM and analyzed by ANOVA, followed by Student-Newman-Keuls test or by Student t test. P<0.05 was considered significant. Maximal ACh-, and sodium nitroprusside–induced responses (Emax) were calculated as maximal percentage increments of lumen diameter. “n” indicates the number of experiments.

Results
Body weight, assessed immediately before animal sacrifice, was similar in all of the groups (Table). Systolic blood pressure was significantly higher in SHRs compared with WKYs, and it was unaffected by atorvastatin. Plasma cholesterol levels were significantly reduced by atorvastatin in both groups. Baseline and NE-induced contraction of vascular lumen diameters did not significantly differ among groups (Table).

Effects of COX-1 and COX-2 Inhibition, TP Receptor Antagonism, and ROS Scavenging on Endothelium-Dependent Relaxation
In WKYs, ACh-induced artery relaxation was not modified by SC-560, Dup-697, SQ-29548, or ascorbic acid. By contrast, SHRs showed an attenuated relaxation to ACh (P<0.001 versus WKYs), which was unaffected by SC-560, but significantly improved, although not normalized,
by Dup-697 ($P<0.05$ versus controls; Figure 1B). The response to ACh was also enhanced by SQ-29548 to similar values to those obtained with COX-2 blockade (Figure 1B). When applied concomitantly, Dup-697 and SQ-29548 failed to further increase ACh-induced relaxation (data not shown). Relaxation to ACh was not affected by ozagrel in WKYs or SHRs (data not shown). In vessels from SHRs, ascorbic acid normalized the ACh-induced relaxation (Figure 1B).

**Effects of NOS Antagonism and NADPH Inhibition on Endothelium-Dependent Relaxation**

In WKY rats, the relaxation to ACh was significantly blunted by L-NAME (inhibition: $-38.9\pm1.1\%$; Figure 2A). Apocynin did not affect the response to ACh or the inhibitory effect of L-NAME (ACh plus L-NAME and apocynin, inhibition: $-39.1\pm1.4\%$; Figure 2A). In SHRs, the inhibition by L-NAME on relaxation to ACh was lower compared with WKYs (inhibition: $-8.1\%$; Figure 2B). Apocynin normalized the endothelium-dependent relaxation and restored the inhibitory effect of L-NAME (ACh plus L-NAME and apocynin, inhibition: $-35.2\pm1.2\%$; Figure 2B). Dup-697 failed to enhance the inhibitory effect of L-NAME (ACh plus L-NAME, inhibition: $-6.9\pm0.8\%$; ACh plus L-NAME and Dup-697, inhibition: $-6.8\pm0.5\%$, Figure 2C).

**Effects of Atorvastatin on Endothelium-Dependent Relaxation**

In vessels from SHRs, endothelial alterations were prevented by treatment with atorvastatin. In particular, the relaxation to ACh was preserved ($E_{max}$: $97.8\pm1.1\%$), and it was no longer affected by SC-560 ($E_{max}$: $98.5\pm1.3\%$), SQ-29548 ($E_{max}$: $98.1\pm1.1\%$), or Dup-697 (Figure 2D). Moreover, both ascorbic acid ($E_{max}$: $98.3\pm0.5\%$) and apocynin failed to modify the relaxation to ACh, and the inhibitory effect of L-NAME was restored (ACh plus L-NAME, inhibition: $-41.1\pm1.2\%$, Figure 2D). Vessels from atorvastatin-treated WKYs responded to ACh in a similar way to untreated controls (ACh, $E_{max}$: $97.5\pm0.3\%$; ACh plus Dup-697, $96.2\pm0.7\%$; ACh plus ascorbic acid, $96.8\pm0.5\%$; ACh plus L-NAME, $58.1\pm0.7\%$; inhibition: $-39.4\pm0.9\%$). Relaxations to sodium nitroprusside were similar in WKYs and SHRs ($E_{max}$: $97.4\pm0.6\%$ and $98.0\pm0.8\%$, respectively), and they were not modified by atorvastatin (WKY: $98.1\pm1.1\%$; SHR: $97.9\pm1.4\%$).

**Effects of Atorvastatin on Vascular Superoxide Anion Generation and Plasma Malondialdehyde Levels**

At baseline, analysis of DHE red fluorescence revealed an increased production of superoxide anion from mesenteric vessels in SHRs, as compared with WKYs (Figure 3). Atorvastatin abrogated superoxide production in SHRs, but not in WKYs (Figure 3). SHRs also displayed high malondialdehyde plasma levels, and such an increment was attenuated by atorvastatin (Table).

**RT-PCR Analysis of COX-2 Expression**

The basal expression of COX-2 mRNA was higher in SHRs than in WKYs (Figure 4). Atorvastatin downregulated vascular COX-2 expression in SHRs to levels similar to those in WKYs. Statin treatment did not affect COX-2 expression in WKYs (Figure 4).
6-keto-PGF₁α and 8-Isoprostane Production

At baseline, the release of 6-keto-PGF₁α was higher in the incubation medium of mesenteric vessels from SHRs as compared with WKYs and it was not affected by Dup-697 (Figure 5). 6-Keto-PGF₁α release was also unaffected by apocynin (from 37.8±11.2 to 34.0±8.6; P value not significant), as well as vessels from atorvastatin-treated SHRs (39.4±12; P value not significant versus control SHRs). At baseline, 8-isoprostane production from mesenteric vessels was higher in SHRs as compared with WKYs, and it was significantly reduced after incubation with the COX-2 inhibitor (Figure 5). 8-Isoprostane production was also significantly decreased by apocynin incubation (from 22.1±6.1 to 11.8±5.4; P<0.05) and, to a similar extent, in vessels from atorvastatin-treated SHRs (10.9±3.2; P<0.05 versus control SHRs).

Western Blot Analysis of pAkt, pERK-1/2, peNOS and iNOS

A reduced pattern of Akt phosphorylation was found in mesenteric vessels from SHRs as compared with control WKYs (Figure 6). Atorvastatin did not affect pAkt levels in WKYs, whereas significant increments were detected in mesenteric vessels from SHRs (Figure 6). The phosphorylation pattern of ERK-1/2 in WKY vessels displayed some variability irrespective of atorvastatin treatment. pERK-1/2 levels in control SHRs were comparable to levels in control WKYs. Atorvastatin enhanced ERK-1/2 phosphorylation in SHRs (Figure 6). Negligible levels of eNOS activating phosphorylation were found in vessels from control WKYs, control SHRs, and atorvastatin-treated WKYs. By contrast, atorvastatin enhanced the activating phosphorylation of eNOS in SHRs while not significantly modifying the pattern of total eNOS expression (Figure 6). iNOS expression was similar in control WKYs, atorvastatin-treated WKYs, and control SHRs. In mesenteric vessels from SHRs, atorvastatin elicited a marked increase in iNOS expression (Figure 6).

Discussion

The present study shows that, in mesenteric resistance arteries from SHRs, a major mechanism of endothelial dysfunction is represented by increased ROS production, leading to reduced NO availability. Both ascorbic acid and apocynin were able to normalize the endothelium-dependent relaxation and to restore the inhibitory effect of L-NAME, thus strengthening the concept that an ROS excess, produced via NADPH oxidase activation, is a major determinant of vascular endothelial alterations in SHRs.1,22,23 Our first major novel finding
consists of the demonstration of COX-2 overexpression and participation of COX-2–derived contracting prostanoids to the impairment of endothelial function in SHR mesenteric vessels. Indeed, Dup-697, but not SC-560, improved the relaxant response to ACh, indicating that COX-2, but not COX-1, contributes to the pathophysiology of endothelial dysfunction in mesenteric arteries from SHRs. In keeping with our functional results, RT-PCR revealed an upregulation of COX-2 expression in vessels from SHRs. In addition, to assess which COX-2–derived mediator might be implicated in these vascular alterations, the involvements of TP receptors and TXA2 were investigated by testing SQ-29548 and oza-grel. In vessels from SHRs, the blunted endothelium-dependent relaxation was improved by TP receptor blockade to similar levels obtained with COX-2 inhibition, whereas the response to ACh was unaffected by TX synthase inhibition. In addition, a possible involvement of vasoconstrictor prostanoids not acting on TP receptors was ruled out by our observation that, under combined COX-2 and TP receptor blockade, no further improvement of endothelium-dependent relaxation could be obtained. Our findings confirm and extend to mesenteric small arteries previous reports that excluded the participation of TXA2 as a COX-derived contracting prostanoid in SHRs.24 On the basis of these observations, we investigated further which contracting prostanoid, other than TXA2, may act as a TP receptor agonist. According to available literature,25 we chose to evaluate 6-keto-PGF1α, the stable metabolite of prostacyclin, because prostacyclin is considered a crucial prostanoid generated by endothelial cells, with a well-recognized physiological role in the control of vascular contractility. Moreover, 8-isoprostane was chosen as a main product of arachidonic acid resulting from mechanisms related to oxidative stress. Indeed, an increased production of 8-isoprostane has been documented in vascular segments from hypertensive animals,6 and its ability to activate contracting TP receptors has been demonstrated.26 In our experiments, 6-keto-PGF1α levels were greater in SHRs as compared with WKYs, and they were not affected by COX-2 inhibition, thus confirming previous findings in SHR aortas that prostacyclin is mainly produced by COX-1.25 This result, together with our functional data, showing that the COX-1 inhibitor failed to affect the ACh-induced relaxation, argues against the possibility that prostacyclin contributes significantly to the control of endothelium-dependent vascular reactivity in SHRs. This proposal is supported by our results obtained with apocynin incubation, which, while normalizing the endothelial function, failed to affect the release of 6-keto-PGF1α. By contrast, 8-isoprostane appears to be a COX-2–derived contracting prostanoid, as documented by its higher levels in SHRs than in WKYs and its significant decrease after incubation with the COX-2 inhibitor. Furthermore, 8-isoprostane production was reduced by incubation with apocynin, thus suggesting that the oxidative status, likely driven by NADPH oxidase activity, may promote the production of contracting prostanoids in the SHR model. Taken together, our results indicate that overexpressed COX-2 in SHRs generates a prostanoid, likely 8-isoprostane, which contributes to mesenteric endothelial dysfunction by inducing a TP-receptor–mediated vasoconstriction (Figure 7). However, our findings do not allow us to exclude the involvement of other endoperoxides, and this possibility requires further investigations.

The present observations agree with and extend previous evidence supporting a contribution of COX-2 to vascular dysfunction in SHRs. Indeed, an enhanced COX-2 expression was observed in aortic segments from SHRs.4,6,7 In addition, in the same animal model, Alvarez et al.6,7 demonstrated an increased participation of COX-2–derived contractile prostanoids in the responses to phenylephrine. Of importance, our experiments also demonstrated that the blunted inhibitory effect of L-NAME on ACh-induced relaxation was not modified by COX-2 blockade in SHRs. These results argue against possible cross-talks between COX-2 and NO and suggest that COX-2–derived 8-isoprostane counteracts the ACh-evoked, endothelium-dependent relaxation independent of the NO pathway.
The second major novel finding of our study deals with the impact of atorvastatin on endothelial dysfunction and NO availability, and its modulation of COX-2 vascular expression and function. After atorvastatin treatment, we observed a normalisation of endothelial function and a restoration of NO availability, as documented by the full inhibition by L-NAME on ACh-induced relaxation. Under these conditions, the ACh-evoked relaxation was no longer sensitive to ROS scavenging, NADPH oxidase blockade, COX-2 inhibition, or TP receptor antagonism. In conjunction with our functional data, the DHE analysis documented that the enhanced generation of vascular superoxide anion in SHRs was dramatically reduced by this statin. In line with these results, the increase in plasma malondialdehyde levels was abrogated by atorvastatin. Moreover, atorvastatin-treated SHRs revealed a marked downregulation of COX-2 expression, together with a blunted 8-isoprostane production. These findings provide the first evidence that atorvastatin is able to abrogate endothelial dysfunction in resistance vessels from SHRs by restoring NO availability, preventing intravascular superoxide anion production, and downregulating the expression of COX-2 and the production of the COX-2–derived contracting prostanoid 8-isoprostane. Previous reports have provided convincing evidence of ROS generation as a major mechanism leading to enhanced NO availability and vasomotor activity in SHRs and in other animal models of vascular disease. Taken together, our results allow us to propose that, beyond the restoration of NO availability, the abrogation of vascular ROS production is a mechanism whereby atorvastatin was able to decrease mesenteric COX-2 expression and activity in SHRs. It is conceivable that, under pathological conditions, such as that occurring in SHRs, COX-2 is switched to convert arachidonic acid into eNOS-dependent NO. Therefore, although previous reports identified 4-hydroxy-2-nonenal as a potential COX-2 inducer, this compound, generated during oxidative stress or lipid oxidation, may contribute to the TP receptor activation and other prostanoids. 

Reduced NO-mediated relaxation

Increased TP receptor-mediated contraction

The lack of effect by the COX-1 inhibitor on the relaxant response to ACh after atorvastatin treatment likely excludes a functional involvement of COX-1 in the modulation of endothelial function. Therefore, although previous reports documented a COX-1/COX-2 compensatory phenomenon in SHRs.31 In our experiments, a marked increment of Akt and iNOS expression in vessels from statin-treated SHRs. These findings provide the first demonstration of a statin-induced Akt-eNOS phosphorylation and iNOS expression via ERK-1/2-Akt phosphorylation, leading to increased NO production.

With respect to the putative molecular mechanisms linking oxidant excess to COX-2 induction, previous experimental reports identified 4-hydroxy-2-nonenal as a potential COX-2 inducer. In particular, it was proposed that this compound, generated during oxidative stress or lipid oxidation, may induce the phosphorylation of p38 mitogen-activated protein kinase, leading to an enhancement of COX-2 expression.29

Figure 7. Schematic diagram showing the proposed mechanisms accounting for endothelial dysfunction in mesenteric resistance vessels from SHRs and the normalizing effects of atorvastatin. In SHRs, an enhanced generation of ROS via vascular NADPH oxidase activation occurs, leading to a reduced NO availability and hyperproduction of peroxynitrites (ONOO⁻). The ROS excess might upregulate COX-2, which is also functionally stimulated by acetylcholine. Under such conditions, COX-2 is switched to convert arachidonic acid into endotheroxides, including 8-isoprostane, which activate the contracting TP receptors. This detrimental cascade is reverted by atorvastatin, which downregulates the activity of NADPH oxidase, leading to a decrease in oxidative stress and related COX-2 induction, and enhances eNOS phosphorylation and iNOS expression via ERK-1/2-Akt phosphorylation, leading to increased NO production.
These results agree with a previous report on mouse myocardial tissues, showing that the statin-induced Akt/eNOS phosphorylation depends partly on ERK1/2 activation. In addition, a study on cultured vascular smooth muscle cells documented that ERK activation is necessary for iNOS induction. Thus, our findings suggest that chronic aterovasculature may promote the activation of different signaling pathways, which concur to generate NO in mesenteric small arteries by activating eNOS-Ser phosphorylation and iNOS induction.

In conclusion, the present study indicates that COX-2–derived 8-isoprostane, acting as a TP receptor agonist, contributes to endothelial dysfunction in SHR mesenteric small arteries without any direct interaction with NO. In this model of hypertension, atorvastatin can normalize endothelium-dependent relaxation through an abrogation of intravascular superoxide anion production, a restoration of NO availability, and a reduction of COX-2 expression and production of the contracting prostanoid 8-isoprostane.

**Perspectives**

By means of a genetic model of hypertension, we have demonstrated that atorvastatin can downregulate COX-2 expression and its production of contracting prostanoids at the level of peripheral resistance arteries, a vascular district where this COX isoform contributes to endothelial dysfunction. This detrimental action of COX-2 has also been described in hypertensive patients and represents one mechanism whereby COX-2 may promote atherosclerosis, as documented in other animal and human models of vascular disease. Taken together, the present findings suggest that vascular COX-2 downregulation represents a prominent mechanism whereby statins may attenuate the development of the atherosclerotic process in normocholesterolemic hypertensive patients.

**Source of Funding**

This work was partly supported by a institutional research grant issued by the Interdepartmental Centre for Research in Clinical Pharmacology and Experimental Therapeutics, University of Pisa.

**Disclosures**

None.

**References**


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Hypertension. published online April 20, 2009; Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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