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Cilostazol enhances atorvastatin-induced vasodilation of female rat aorta during aging

KE Nurullahoğlu-Atalık[1], S Kutlu[2], H Solak[2], R Özen Koca[2]

[1]Department of Pharmacology, Faculty of Meram Medicine, University of Necmettin Erbakan, Konya, Turkey
[2]Department of Physiology, Faculty of Meram Medicine, University of Necmettin Erbakan, Konya, Turkey

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Statins have cholesterol-independent effects including an increased vascular nitric oxide activity and are commonly used by patients with cardiovascular disease. Such patients frequently have cardiovascular diseases, which may be treated with cilostazol, a platelet aggregation inhibitor. This study was designed to investigate whether combined use of cilostazol would increase the inhibitory effect of statin on vascular smooth muscle and how maturation would affect these responses. Female Wistar rats, aged 3–4 months (young) and 14–15 months (adult), were sacrificed by cervical dislocation and the thoracic aorta was dissected and cut into 3- to 4-mm-long rings. The rings were mounted under a resting tension of 1 g in a 20-ml organ bath filled with Krebs–Henseleit solution. Rings were precontracted with phenylephrine (10^{-6} M), and the presence of endothelium was confirmed with acetylcholine (10^{-6} M). Then, the concentration–response curves were obtained for atorvastatin alone (10^{-10} to 3×10^{-4} M; control) and in the presence of cilostazol (10^{-6} M) in young and adult rat aortas. This experimental protocol was also carried out in aorta rings, which had been pretreated with N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M). Atorvastatin induced concentration-dependent relaxations in young and adult rat thoracic aorta rings precontracted with phenylephrine. The pIC_{50} value of atorvastatin was significantly decreased in adult rat aortas. In addition, pretreatment of aortas with cilostazol enhanced the potency of atorvastatin in both young and adult aortas. Incubation with L-NAME did not completely eliminate the relaxations to atorvastatin in the presence of cilostazol. These results suggest that combined application of cilostazol with atorvastatin was significantly more potent than atorvastatin alone. Combined drug therapy may be efficacious in delaying the occurrence of cardiovascular events.

Keywords: age, atorvastatin, cilostazol, rat aorta, nitric oxide, relaxation

Introduction

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, have been broadly used in clinical practice owing to both its potent lipid-modifying effects and other cardioprotective effects including increasing nitric oxide production, improving inflammation and oxidation, enhancing endothelial progenitor cells migration, and so on which now are referred to as pleiotropic effects (4, 5, 28). Statins are widely used and are the most effective hypolipidemic agents available for the reduction of cardiovascular risks and prevention of the worsening of disease/disability in patients with established cardiovascular disease (secondary prevention). Statins have cholesterol-independent effects including an increased vascular nitric oxide activity and are commonly used by patients with cardiovascular disease. Such patients frequently have cardiovascular diseases, which are treated with cilostazol, a platelet

Corresponding author: K. Esra Nurullahoğlu-Atalık, PhD
Department of Pharmacology, Faculty of Meram Medicine, University of Necmettin Erbakan
Konya 42080, Turkey
Phone: +90 332 223 79 56; Fax: +90 332 223 79 52; E-mail: esraatalik@hotmail.com

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aggregation inhibitor. However, to the best of our knowledge, there are no studies that analyze the combined effects of atorvastatin and cilostazol on vascular smooth muscle reactivity.

Maturation and senescence are physiological process. The effects of biological age on the responsiveness of the vascular system have been widely studied in terms of both maturation and senescence. Despite numerous recent studies have aimed to determine the effects of age on vascular reactivity of different agents, studies with statins remain incomplete and there are no *in vitro* studies that analyze the effects of statins on age-dependent responses of the rat aorta. Gong et al. (9) reported that the long-term administration of atorvastatin improves age-related endothelial dysfunction in aged rat aorta. Furthermore, Alvarez de Sotomayor et al. (2) have demonstrated that oral administration of simvastatin improves endothelial dysfunction in the aorta from aged rats by mechanisms associated with enhanced nitric oxide vasodilatation, reduced release of thromboxane A_2 from cyclooxygenase, and increased antioxidant properties of the vessel wall. These data underscore a new therapeutic perspective for simvastatin in age-related endothelial dysfunction. These results are all obtained in models, which used oral administration of statins.

Since its discovery in the 1980s, nitric oxide is in fact the elusive endothelium-derived relaxing factor, it has become evident that nitric oxide is not only a major cardiovascular signaling molecule, but changes in its bioavailability are crucial in the development of atherosclerosis. Sustained high levels of harmful circulating stimuli associated with cardiovascular risk factors elicit responses in endothelial cells that appear sequentially, namely endothelial cell activation and endothelial dysfunction. Endothelial dysfunction, characterized by reduced nitric oxide bioavailability, is now recognized by many as an early, reversible precursor of atherosclerosis. It is known that the ability of the endothelium to produce nitric oxide is reduced with aging (29).

Cilostazol, used as a selective inhibitor of type III phosphodiesterase (PDE3), induces an antiplatelet and antithrombotic effect by increasing cyclic adenosine monophosphate (cAMP) in platelets and vascular smooth muscle (15). Recently, Li et al. (17) reported that cilostazol induced the relaxation of rabbit thoracic aorta through activation of the big-conductance Ca^{2+}-activated K^+ channel via an endothelium-independent, protein kinase A-dependent signaling pathway. No previous study has focused on the effect of aging on the vasodilator effects of atorvastatin and also possible interaction of cilostazol with statins *in vitro*. However, an interaction could result from the fact that both drugs activate the nitric oxide–cyclic guanosine monophosphate pathway, thereby producing vasodilation. This study was designed to determine the effect of age on the statin-induced relaxation of rat thoracic aorta, and to identify whether pretreatment with cilostazol affects the vascular reactivity to atorvastatin. We also examined the role of nitric oxide in this interaction.

Materials and Methods

Animals and husbandry

Female Wistar rats, aged 3–4 months (young) and 14–15 months (adult), were obtained from Application and Research Center of Experimental Medicine, Necmettin Erbakan University (Konya, Turkey). The protocols of the animal experiments were approved by the internal ethical committee of Application and Research Center of Experimental Medicine, Necmettin Erbakan University.

Experimental design

Rats were sacrificed by cervical dislocation and the descending thoracic aorta was quickly isolated, cleaned, and sectioned into 3- to 4-mm-long rings. The aorta rings were mounted under a resting tension of 1 g in a 15-ml organ bath filled with Krebs–Henseleit solution [composed of (mM): NaCl 119, KCl 4.70, $MgSO_4$ 1.50, KH_2PO_4 1.20, $CaCl_2$ 2.50, $NaHCO_3$ 25, Glucose 11], maintained at 37 °C, and aerated with 95% O_2 and 5% CO_2. Tissues were allowed to equilibrate for 1 h. Changes in isometric tension of aortic rings were recorded using a four-channel force-displacement transducer (BIOPAC MP36, Santa Barbara, CA, USA) connected through amplifiers to a ITBS08 Integrated Tissue Bath System (Commat, Ankara, Turkey).

Adequate care was taken to insert the hooks without damaging the endothelium. After the stabilization period, isometric contraction was induced by phenylephrine (10^{-6} M). During the tonic phase of the contraction, acetylcholine (10^{-6} M) was added to verify the integrity of the endothelium. The vascular endothelium was considered intact when the aortic rings showed relaxation ≥50%, and non-functional if relaxation was ≤10%. After washing, the tonic component of a second reaction was induced by phenylephrine (10^{-6} M) for 30 min, followed by the cumulative addition of atorvastatin (10^{-10} to 3×10^{-4} M) to the organ baths to induce relaxation.

After the first concentration–response curve was completed, rings were washed and allowed to reestablish resting tension. A second concentration–response curve to atorvastatin (10^{-10} to 3×10^{-4} M) was determined in the presence of cilostazol (10^{-6} M). Cilostazol was added to organ baths and the tissues were allowed to equilibrate for 30 min. Cilostazol and atorvastatin were prepared daily.

The influence of nitric oxide on relaxations to atorvastatin was specifically addressed by pretreating the rings with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M; 24). L-NAME had been added to the organ bath 20 min before concentration–response curves were obtained.

Statistical analysis

Values are given as mean ± SD. In all experiments, "*n*" is the number of rats from which the aortas were obtained. Relaxation responses to atorvastatin were expressed as percentages of the phenylephrine-induced contraction (10^{-6} M). The concentrations of atorvastatin causing 50% of the maximal response (IC_{50}) were calculated from each individual concentration–response curves. pIC_{50} ($-\log IC_{50}$) values for atorvastatin (control and cilostazol-pretreated) curves obtained were compared using Student's *t*-test. Statistical significance was set at $p < 0.05$.

Drugs

Phenylephrine, acetylcholine (dissolved in distilled water), cilostazol, and atorvastatin [dissolved in dimethyl sulfoxide (DMSO)] were used. The concentration of DMSO in the tissue bath was always kept below 0.4%. Phenylephrine and acetylcholine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cilostazol and atorvastatin were kindly provided by Abdi İbrahim Drug Industry (Istanbul, Turkey). DMSO was determined to have no effect on phenylephrine-induced contractions.

Results

Atorvastatin (10^{-10} to 3×10^{-4} M) induced relaxation in a concentration-dependent way in young and adult rat aortic rings precontracted with phenylephrine (Fig. 1). In young rat

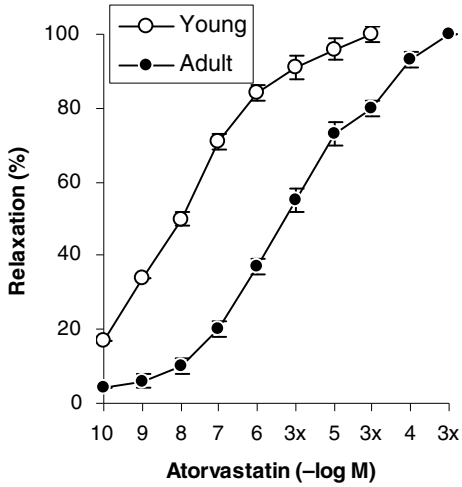

Fig. 1. Concentration–response curves showing relaxations induced by atorvastatin (10^{-10} to 3×10^{-4} M) in young and adult rat thoracic aorta. Each point represents the mean ± SD expressed as a percentage of the tension developed by 10^{-6} M phenylephrine. Each value is derived from six experiments

aortas, the relaxation response to atorvastatin was significantly greater than in the aortas of adult rats. The sensitivity of atorvastatin was significantly decreased in mature rat aortas ($pIC_{50} = 7.9 \pm 0.1$ in young and 5.6 ± 0.1 in adult rats, $p < 0.05$).

After incubation with the selective PDE3 inhibitor cilostazol (10^{-6} M), atorvastatin evoked sustained relaxations of young and adult rat thoracic aorta rings in a concentration-dependent manner. In the presence of cilostazol, atorvastatin induced relaxation in low concentrations. Treatment with cilostazol significantly enhanced the potency of atorvastatin compared with control at both ages. Atorvastatin produced 80% relaxation at 10^{-10} M concentration in the presence of cilostazol in both age groups.

Both in young and adult aortas, preincubation with the non-selective nitric oxide synthase inhibitor L-NAME suppressed atorvastatin-induced relaxation (Figs 2 and 3). Incubation of aortic rings with L-NAME in the presence of cilostazol did not completely

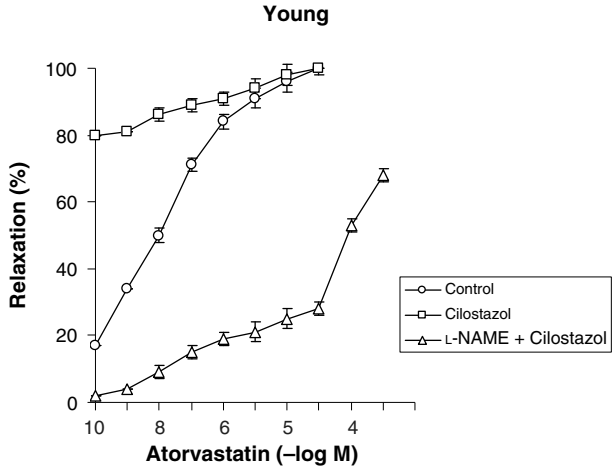

Fig. 2. Concentration–response curves showing relaxations induced by atorvastatin (10^{-10} to 3×10^{-4} M) in young rat thoracic aorta, in the presence of cilostazol (10^{-6} M) and cilostazol (10^{-6} M) with L-NAME (10^{-4} M). Each point represents the mean ± SD expressed as a percentage of the tension developed by 10^{-6} M phenylephrine. Each value is derived from six experiments

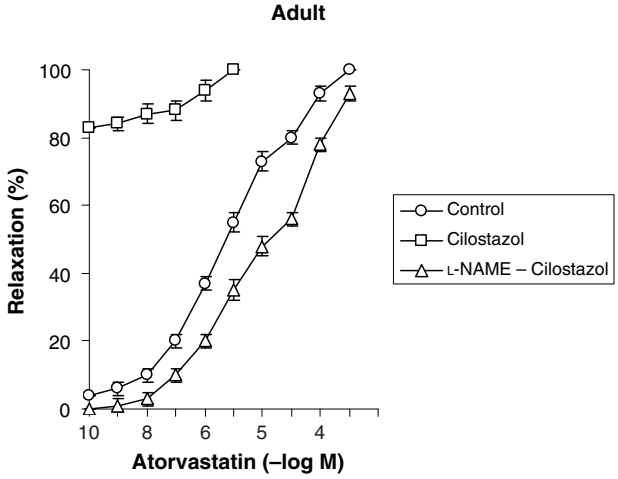

Fig. 3. Concentration–response curves showing relaxations induced by atorvastatin (10^{-10} to 3×10^{-4} M) in adult rat thoracic aorta, in the presence of cilostazol (10^{-6} M) and cilostazol (10^{-6} M) with L-NAME (10^{-4} M). Each point represents the mean ± SD expressed as a percentage of the tension developed by 10^{-6} M phenylephrine. Each value is derived from six experiments

eliminate the relaxation to atorvastatin but significantly reduced the potency of atorvastatin-induced relaxation in both age groups. In L-NAME-preincubated rings, pIC_{50} values decreased to 4.1 ± 0.6 in young and 4.9 ± 0.6 in adult rats ($p < 0.05$). Preincubation with L-NAME in the presence of cilostazol diminished the vasodilator response to atorvastatin. The maximal relaxations are $68\% \pm 3.8$ and $93\% \pm 4.1$ in young and adult rat aortas, respectively.

On the other hand, we also determined that the concentration–response to atorvastatin was reproducible (time-match control experiments) and did not change in the presence of the vehicle of atorvastatin (DMSO), in terms of both maximum and pIC_{50} values (data not shown).

Discussion

In this study, atorvastatin induced concentration-dependent relaxations in young and adult rat thoracic aorta rings precontracted with phenylephrine. The sensitivity of atorvastatin was significantly decreased in adult rat aortas, and cilostazol pretreatment enhanced the potency of atorvastatin. It has been reported that statins are also able to induce a vasodilation, which may be endothelium-dependent and induced by an increased production of nitric oxide (2). It is known that cilostazol and other cAMP-elevating agents increase nitric oxide production by endothelial cells (24). In this study, we observed cilostazol potentiated atorvastatin-induced relaxation for the first time.

Beyond their primary mode of action of reducing plasma cholesterol levels, statins have beneficial effects on the cardiovascular system. Several studies have revealed that statins enhance the bioavailability of nitric oxide and consequently improve endothelial function (6, 7, 12, 18, 25, 31). We know that some factors, including age, influence the response of vascular smooth muscle to exogenous agents. However, no previous *in vitro* data on the effects of atorvastatin of rat thoracic aorta during aging or maturation have been published. Zhang et al. (32) reported that atorvastatin can increase endothelial nitric oxide synthase synthesis in the vital organs of aging rats, which partially explains the organ-protective effect of atorvastatin against myocardial ischemia–reperfusion.

Our results indicate that atorvastatin induced concentration-dependent relaxation of young and adult phenylephrine-contracted rat aortas. Phenylephrine, an alpha-adrenergic agonist, contracts smooth muscle cells through the extracellular Ca^{2+} influx in receptor-operated Ca^{2+} channels and through the release of internal Ca^{2+} from specific inositol 1,4, 5-trisphosphate receptor channels in the sarcoplasmic reticulum membrane (13, 19). In this study, atorvastatin inhibited the contraction induced by phenylephrine in a concentration-dependent manner. This result suggests that atorvastatin can inhibit the vasocontraction induced by extracellular Ca^{2+} entry via the receptor-operated Ca^{2+} channel pathway. Some studies have demonstrated that simvastatin (1, 3, 27) and atorvastatin (27) also directly relax vascular smooth muscle by inhibiting Ca^{2+} influx through voltage-operated Ca^{2+} channels and Ca^{2+} release from intracellular store site. Tesfamariam et al. (27) reported that atorvastatin may have additional effects on Ca^{2+} regulation in vascular smooth muscle that are not due to cholesterol depletion. They also observed that simvastatin and atorvastatin may reduce agonist-stimulated rise in $[Ca^{2+}]_i$ by inhibiting Ca^{2+} influx. It is known that simvastatin and atorvastatin are lipophilic and freely diffuse into cells (11). These findings support our results. Moreover, atorvastatin was more potent in the young than in the adult group in this study. However, the mechanisms for the decreased sensitivity to atorvastatin are unknown. Chronic and minor pathological changes of the vessel morphology during aging might affect the potency of atorvastatin in rat aorta. To the best of our knowledge, this is the first *in vitro* study to show the effects of age on atorvastatin-induced relaxations of isolated rat aorta. The maximal plasma concentration of atorvastatin is reported to be 252 μM in patients treated with the recommended oral doses (80 mg; 16). Thus, in this study, the lower concentrations of atorvastatin (10^{-10} to 3×10^{-4} M), which initiate acute relaxant response in rat aorta, correspond to plasma levels of this statin derived from commonly prescribed doses.

Cilostazol is an inhibitor of PDE3 that increases the levels of cAMP and the active form of protein kinase A, a process closely related to the inhibition of platelet aggregation and relaxation of vascular smooth muscle (15). In this study, we also investigated the role of cilostazol pretreatment on the atorvastatin-induced responses. Interestingly, pretreatment with cilostazol enhanced the potency of atorvastatin in both young and adult rat aortas. Furthermore, in adult aortas, cilostazol enhanced the potency more potently than in the young group. Based on our data, we cannot suggest which mechanisms underlie the increased sensitivity to atorvastatin in the presence of cilostazol. Previously, we reported that cumulative addition of cilostazol caused concentration-dependent relaxations of rat thoracic aorta rings (23). However, no previous study has addressed the possible vascular interactions of statins with cilostazol in isolated rat aorta. Cilostazol has many pharmacological actions, including vasodilation, inhibition of platelet activation and aggregation, inhibition of thrombosis, increased blood flow to the limbs, improvement in serum lipids with lowering of triglycerides and elevation of high-density lipoprotein cholesterol, and inhibition of growth of vascular smooth muscle contractions (8, 14, 15). We know that statins are commonly used by patients with cardiovascular diseases. Such patients frequently have cardiovascular diseases, which may be treated with cilostazol. Nakamura et al. (21) have previously reported the protein kinase A-independent vasodilating effect of PDE3 inhibition using pressurized rabbit cerebral penetrating arterioles. It is expected that the effectiveness of vasodilation by PDE3 inhibition is greatly dependent on the type of the blood vessel sampled, the agonist stimulation, and the timing of drug treatment. Cilostazol is clinically applicable to

the patients with intermittent claudication and atherosclerosis obliteration. It is generally thought that endothelium-dependent vasodilation and capillary-like formation underlie the protection of PDE3 inhibition against peripheral arterial disease. Nakamura et al. (22) reported that cilostazol-induced vasodilation of the rat thoracic aorta was dependent on the endothelium, which released nitric oxide from aortic endothelial cells. Furthermore, Li et al. (17) reported that cilostazol induced the relaxation of rabbit thoracic aorta through activation of the big-conductance Ca^{2+}-activated K^+ channel via an endothelium-independent, protein kinase A-dependent signaling pathway. These investigators also suggested a cilostazol-induced endothelium-independent relaxation, since the relaxations of cilostazol on endothelium-intact and endothelium-denuded arteries were not different. In this study, incubation of aortic rings with L-NAME in the presence of cilostazol did not completely eliminate the relaxation to atorvastatin but significantly reduced the responses of this statin in young and adult rat aortas, especially in young rats. There appears to be a nitric oxide-independent effect of atorvastatin since L-NAME did not totally abolish atorvastatin-induced relaxation. Similarly, Sönmez Uydeş-Doğan et al. (26) reported that pravastatin, atorvastatin, and cerivastatin can acutely induce vasorelaxation on precontracted aortic rings via both endothelium-dependent and -independent mechanisms. The E_{max} and IC_{50} values that the researchers found were different from those of our work. The reason for this discrepancy may be related to differences between contractile agents used, ages or sex of rats, and perhaps working conditions. In that study, nitric oxide release seems to play a more dominant role in atorvastatin responsiveness as determined by a higher inhibition of maximal relaxation by nitric oxide synthase inhibitor N^G-nitro-L-arginine (L-NOARG). Recently, Meschiari et al. (20) reported that statins exert pleiotropic effects independent of cholesterol concentrations, including upregulation of nitric oxide formation and matrix metalloproteinase (MMP) downregulation. However, statins also increase tissue concentrations of nitrites, which activate new signaling pathways independent of nitric oxide. Atorvastatin increased nitrite concentrations and nitrite inhibited MMP-9 production by endothelial cells. Furthermore, Guimarães et al. (10) reported that treatment with atorvastatin significantly increased nitrite concentrations in the aortas from hypertensive animals. But we have no explanation about this part of the response because it is not possible to measure vascular nitrite concentrations in our lab. Further studies should be carried out to clarify atorvastatin increasing nitrite mechanisms.

In conclusion, combined drug therapy may be efficacious in delaying the occurrence of cardiovascular events. The results of Wang et al. (30) support our hypothesis that combined treatment with atorvastatin + probucol + cilostazol (APC) significantly attenuates atherosclerosis through inhibiting anti-inflammatory and antioxidant properties independent of the lipid-lowering function. Although it remains to be verified clinically whether combined APC treatment exhibits a "potent antiatherogenic function," it seems that APC more strongly attenuates the progression of atherosclerosis than statin alone in cholesterol-fed rabbits. The investigators also reported that these insights may provide a new concept with which the occurrence of cardiovascular events can be effectively delayed by APC-combined drug treatment in the early stages in patients with or without established atherosclerotic cardiovascular disease. This finding is in line with ours. This study supports that the new concept of statin–cilostazol combination could be valuable in cardiovascular prevention especially during aging. In this study, we used only female rats; therefore, further studies must be performed to clarify the gender difference.

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