FLUVASTATIN IMPROVES VASCULAR FUNCTIONS IN RABBIT CAROTID ARTERIES LOADED WITH OXIDATIVE STRESS

FLUVASTATİN OKSİDATİF STRES ALTINDAKİ TAVŞAN KAROTİD ARTER DAMAR FONKSİYONLARINI İYİLEŞTİRİR

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ABSTRACT

   Oxidative stress is implicated in most cardiovascular diseases and reactive oxygen species (ROS) have a major role in vascular endothelial cell signal transduction. 3-hydroxy-3-methylglutaryl coenzyme A(HMG-CoA) reductase inhibitors including fluvastatin do not only lower plasma cholesterol but also have non-cholesterol lowering (direct) effects on the vessel wall which decrease cardiovascular complications. The effects of fluvastatin are investigated in isolated rabbit carotid artery subjacent diethyldithiocarbamic acid (DETCA)-generated oxidative stress and in relation to nitric oxide synthase inhibition by N^{ω}-nitro L-arginine (L-NA) on responses. Four arterial segments were used from each rabbit. The rings were subjected to reactive oxygen species by incubation with DETCA (3mM) for 30 minutes. Fluvastatin was added to bath 10 minutes before DETCA incubation. L-NA ($10^{-4}M$) was used in one of the organ bath. The relaxant response to acetylcholine (ACh) and contractile reponses to phenylephrine (PE) and serotonin (5HT) were determined. Contractility and sensitivity to serotonin in vessels were not affected by DETCA. Treatment with DETCA diminished the maximum relaxation and sensitivity to ACh. These effects were prevented by fluvastatin. Incubation with L-NA reversed the increased ACh-relaxation induced by fluvastatin. Maximum contractile responses and sensitivity to PE were attenuated in DETCA-treated rings. Fluvastatin normalized the decreased PE contractility caused by DETCA. In conclusion, fluvastatin improves vascular functions under oxidative stress independent of lipid lowering in isolated carotid artery.

   Key Words: *oxidative stress, fluvastatin, N^{ω}-nitro L-arginine (L-NA), vascular reactivity*

ÖZET

Oksidatif stres kardiyovasküler hastalıkların pek çoğuyla ilişkilidir ve reaktif oksijen türleri (ROS) damar endotel hücrelerinin sinyal transdüksiyonunda önemli bir role sahiptir. Fluvastatinin dahil olduğu 3-hidroksi-3 metil koenzim A (HMG-CoA) redüktaz inhibitörleri sadece plazma kolesterolünü düşürmekle kalmaz aynı zamanda da damar duvarı üzerinde kardiyovasküler komplikasyonları azaltan kolesterol düşürücü etkiden bağımsız direkt etkilere de sahiptir. Fluvastatinin dietiltiyokarbamik asit (DETCA) ile oluşturulan oksidatif strese maruz kalmış izole tavşan karotid arterindeki etkileri ve yanıtlar üzerinde N^{ω}-nitro L-arjinin (L-NA) ile nitrik oksit inhibisyonun ilişkisi araştırılmıştır. Her bir tavşandan dört arter segmenti kullanıldı. Ringler, 3mM DETCA ile 30 dakika inkübe edilmek suretiyle reaktif oksijen türlerine maruz bırakıldılar. Fluvastatin, DETCA inkübasyonundan 10 dakika önce banyoya ilave edildi. Organ banyosunun birinde $10^{-4}M$ L-NA kullanıldı. Asetilkoline (ACh) karşı gevşeme yanıtları ile fenilefrin (PE) ve serotonine (5-HT) kasılma yanıtları araştırıldı. Damarlarda serotonine karşı kasılmalar ve duyarlılık DETCA'dan etkilenmedi. DETCA ile tedavi ACh gevşemeleri ve duyarlığı azalttı. Bu etkiler fluvastatin ile engellendi. L-NA ile inkübasyon fluvastatin tarafından oluşturulan ACh gevşemelerindeki artışı geriye döndürdü. DETCA tedavisi gören ringlerde PE maksimum kasılma yanıtları ve duyarlığı azaldı. Fluvastatin DETCA'nın neden olduğu PE kasılma yanıtlarındaki azalmayı normalize etti. Sonuç olarak, fluvastatin izole karotid arterinde oksidatif stres altında bozulan damar fonksiyonlarını lipid düşürücü etkisinden bağımsız olarak iyileştirmektedir.

Anahtar Kelimeler: oksidatif stres, fluvastatin, N^{ω}-nitro L-arjinin (L-NA), vasküler reaktivite

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INTRODUCTION

The role of HMG-CoA reductase inhibitor drugs (statins) in the reduction of serum lipids has been well documented but recently evidence that statins may positively improve many organ systems and diseases beyond lipid lowering effects has assumed greater importance. The term "pleiotropic effects" has been used to explain these properties. Statins affect many metabolic pathways and organ systems, so may have a positive impact on the treatment of many diseases such as atherosclerosis, congestive heart failure, nephropathy, central nervous system diseases, autoimmune diseases, sepsis, gastrointestinal diseases and osteoporosis (1,2). Fluvastatin, a HMG-CoA reductase inhibitor, has been reported to suppress atherosclerotic progression in animal experimental models and in patients with coronary heart disease. It has a characteristic antioxidant property and can protect low-density lipoprotein from oxidative modification in vivo and in vitro (3-6).

Reactive oxygen species (ROS), especially superoxide anion play a major role in the etiology of a wide variety of diseases including diabetes mellitus, atherosclerosis, and hypertension. It has been generally accepted that the vascular system is the first target of ROS. Imbalance between the production of superoxide anion and nitric oxide in the vessel wall has been thought to impair endothelium-dependent vasodilator responsiveness in different models of experimentally induced oxidative stress. However, contractile responses induced by free radicals in vascular system need to be evaluated because of the contradictory results in the literature (7-10). The superoxide dismutases (SOD) represent a major cellular defense against superoxide anion. Three isoenzymes have been identified, including a cytosolic copper/zinc-containing form (Cu/ZnSOD), a mitochondrial manganese-containing form (MnSOD), and an extracellular isoenzyme (ecSOD), which is also a copper/zinc containing enzyme. In the vessel wall, this enzyme binds to endothelium and connective tissue matrix. One third to one half of total SOD activity is made up by ecSOD (11). The experimental use of Cu/Zn SOD inhibitor, DETCA to generate endogenous superoxide anion stress, is well established in both visceral and vascular smooth muscle (12,13). Therefore, in the present study, conditions of oxidative stress were created in isolated carotid artery rings by inactivating endogenous Cu/Zn SOD with DETCA.

The aim of this study was to investigate the effects of DETCA and a possible antioxidant role of fluvastatin on vascular endothelial function and contractile responses in rabbit isolated carotid artery exposed to oxidant stress.

MATERIALS AND METHODS

The animal experiments were carried out in accordance with guidelines described by the Ethics Committee of the Faculty of Pharmacy, Ege University.

Carotid arteries were obtained from white rabbits of either sex (2-2.5 kg) that were killed with an overdose of sodium pentobarbital (i.v.) into the marginal ear vein. After the surrounding fat and connective tissue had been carefully cleaned off, the artery was cut into 4 adjacent pairs of rings 3-4 mm long. Care was taken not to cause damage to the endothelium. Then the rings were suspended in organ chambers filled with physiological salt solution (Krebs) at 37^0, continuously oxygenated with $95\%O_2$ - $5\%CO_2$. The composition of Krebs solution was (in mM): NaCl, 118; KCl, 4.7; $CaCl_2.2H_2O$, 2.5; KH_2PO_4, 1.20; $MgSO_4.7H_2O$, 1.17; Glucose, 11.1; $NaHCO_3$, 25. A resting tension of 2.5 g was applied to rings which were then allowed to equilibrate for 45 minutes before experimental procedures were initiated. In this period tissues were washed out with Krebs

every 15 minutes. The effects of irreversible endogenous superoxide dismutase (SOD) inhibition on vascular functions were investigated by pretreatment with DETCA (3 mM) for 30 min. Fluvastatin ($3x10^{-5}$M) was added to bath before a 10 minute DETCA incubation. 10^{-4}M L-NA (N^{ω}-nitro-L-arginine, nitric oxide synthase inhibitor, Acros Organics®) was added to organ bath at the same time as DETCA. In the four organ baths, experimental groups were as follows: Control ring *[C]*; the ring treated with DETCA *[D]*; the ring treated with DETCA and fluvastatin *[DF]*; the ring subjected to DETCA, fluvastatin and LNA *[DFL]*. Isometrical changes in tension were recorded on Biopack Computer Program (Commat, Ankara, Turkey) via Grass FT3 force transducer.

At the end of the equilibration period, the rings were contracted with potassium chloride (KCl; 60 mM) (Merck®). Maximal contractions for each ring were obtained by reaching the stable plateau value. Following this, the rings were contracted by phenylephrine ($3x10^{-6}$ M) and when a steady tension level was reached, acetylcholine (Merck®) was added in a cumulative manner (10^{-9}–10^{-4} M). Then cumulative phenylephrine (10^{-9}–10^{-4} M) and serotonin (5-HT) (10^{-9}-$3x10^{-5}$ M) dose-response curves were constructed in each ring. After each concentration-response curve, the organ baths were repeatedly washed out and the tissues were allowed to re-equilibrate for 30 min before further experimentation.

All data are expressed as means ± s.e.m; n indicates the number of animals. Statistical comparisons were performed with Tukey's Multiple Comparison Test. Significance was accepted at *P= 0.05*. The negative logarithm of the concentration (pD$_2$) that produced half of the maximal effect (E$_{max}$) of that agonist was calculated using linear regression analysis (Prism 3.02). Means of pD$_2$ and E$_{max}$ values were compared. Acetylcholine-induced relaxations were normalized to the initial phenylephrine contraction. Contractile responses to phenylephrine and serotonin were normalized to the maximum tension induced by KCl for each ring.

RESULTS AND DISCUSSION

*Effects of DETCA and fluvastatin on endothelium-dependent relaxations*

Acetylcholine (ACh) induced concentration-dependent relaxations in control carotid artery rings precontracted with phenylephrine ($3x10^{-6}$M). Maximum responses (E$_{max}$) to Ach were significantly attenuated and, as reflected by pD$_2$ values, concentration-response curve shifted to the right in rings incubated with DETCA. Pretreatment with fluvastatin prevented impaired ACh relaxations and sensitivity in rings under oxidative stress. Inhibition of nitric oxide synthase by L-

NA decreased recovery of maximum relaxations by fluvastatin without affecting the sensitivity in DETCA and fluvastatin together (Figure 1, Table 1).

Effects of DETCA and fluvastatin on contractions

Phenylephrine (PE) induced concentration-dependent contractions in control rabbit carotid artery rings. Incubation with DETCA significantly diminished maximum contractile responses and sensitivity to PE. Fluvastatin pretreatment reversed the inhibitory action of DETCA on maximum contractile responses without affecting sensitivity to PE. Furthermore, L-NA was unaffected by the fluvastatin-mediated enhancement in contractile responses to PE (Figure 2, Table 1).

Serotonin induced concentration-dependent contractions in control rabbit carotid artery rings. DETCA tended to decrease maximum serotonin contractions similar to those observed in PE contractions but this effect did not reach a statistically significant level. Neither the E_{max} nor pD_2 values of serotonin were significantly affected by fluvastatin treatment in rings under oxidative stress. The contractile responses of the rings to serotonin were not found to be significantly different in the presence of inhibition of NO by L-NA (Figure 3, Table 1).
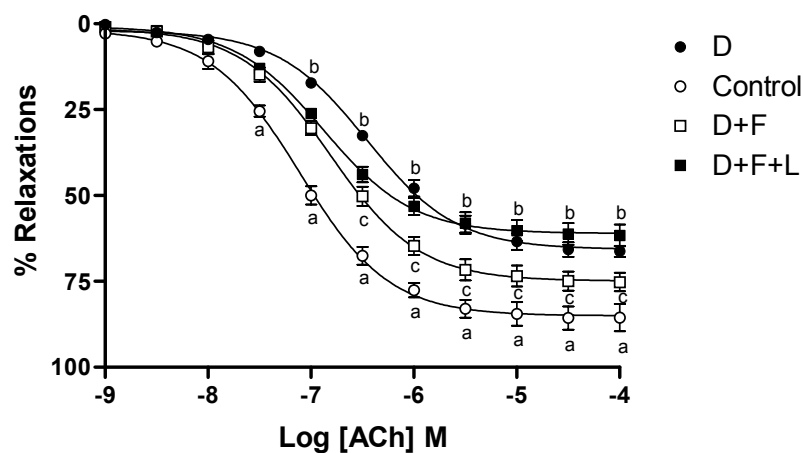
Figure 1: Concentration response curves of acetylcholine in control and treated isolated carotid artery rings (n=5 for each group). *C*: Control ring (○); *D*: DETCA-incubated ring (●); *D+F*: DETCA+fluvastatin-incubated ring (□); *D+F+L*: DETCA+fluvastatin+L-NA-incubated ring (■). Bath final concentration for DETCA (3 mM), fluvastatin (3×10^{-5} M) and L-NA (10^{-4} M). The responses are shown as means±s.e.m. and expressed as % of initial contraction to phenylephrine (3×10^{-6} M). ([a]$P<0.001$, control vs DETCA; [b]$P<0.05$, DETCA vs DETCA + fluvastatin; [c]$P<0.05$, DETCA + fluvastatin vs DETCA + fluvastatin + L-NA, Tukey's Multiple Comparison Test).
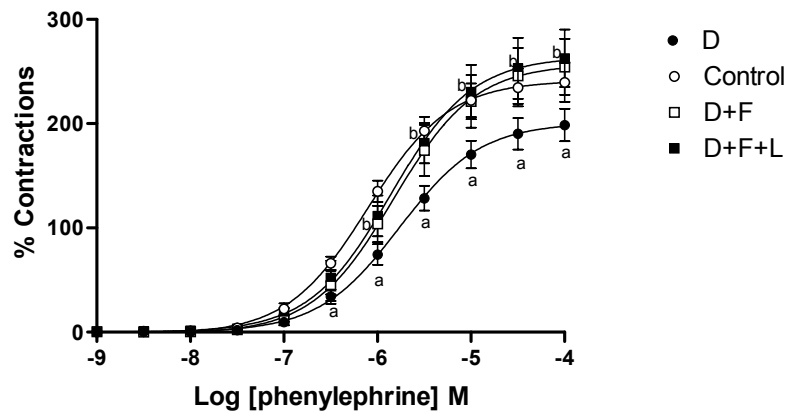
Figure 2: Concentration response curves of phenylephrine in control and treated isolated carotid artery rings (n=5 for each group). *C*: Control ring (○); *D*: DETCA-incubated ring (●); *D+F*: DETCA+fluvastatin-incubated ring (□); *D+F+L*: DETCA+fluvastatin+L-NA-incubated ring (■). Bath final concentration for DETCA (3 mM), fluvastatin (3×10^{-5} M) and L-NA (10^{-4} M). Contractile responses to phenylephrine were normalized to the maximum tension induced by KCl for each ring. ([a]$P<0.05$, control vs DETCA; [b]$P<0.05$, DETCA vs DETCA + fluvastatin; Tukey's Multiple Comparison Test).
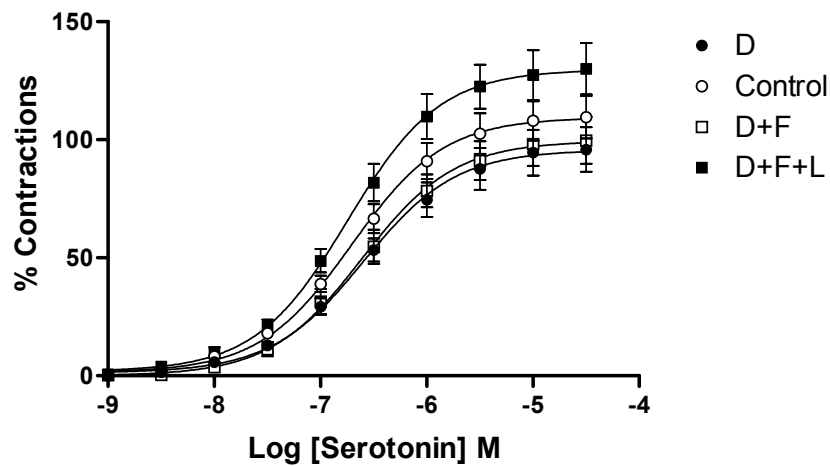
Figure 3: Concentration response curves of serotonin in control and treated isolated carotid artery rings (n=5 for each group). *C*: Control ring (○); *D*: DETCA-incubated ring (●); *D+F*: DETCA+fluvastatin-incubated ring (□); *D+F+L*: DETCA+fluvastatin+L-NA-incubated ring (■). Bath final concentration for DETCA (3 mM), fluvastatin (3×10^{-5} M) and L-NA (10^{-4} M).

Contractile responses to serotonin were normalized to the maximum tension induced by KCl for each ring.

Table 1: The E_{max} and pD_2 values of the concentration-response curves obtained by acethylcholine, phenylephrine and serotonin in groups of isolated carotid artery ring. Shown are means±s.e.m. Five rabbits were used for each experimental groups. *P<0.001 control vs DETCA, +P<0.05 DETCA vs DETCA+fluvastatin, #P<0.05 DETCA+fluvastatin vs DETCA+fluvastatin+L-NA, Tukey's Multiple Comparison Test.

E_{max}	ACETHYLCHOLINE	PHENYLEPHRINE	SEROTONIN
	% of phenylephrine precontraction	% of max.tension induced by KCl	% of max.tension induced by KCl
Control	85.63 ± 9.00*	239.6 ± 18.82*	109.5 ± 9.03
DETCA	66.32 ± 3.65$^{+, \#}$	198.7 ± 15.55^{+}	95.85 ± 9.42
DETCA+fluvastatin	75.26 ± 6.11	254.5 ± 26.82	99.72 ± 9.97
DETCA+fluvastatin+L-NA	61.68 ± 7.06	262.7 ± 27.56	130.1 ± 10.92
pD_2			
Control	7.10 ± 0.19*	6.10 ± 0.07*	6.72 ± 0.13
DETCA	6.46 ± 0.08^{+}	5.72 ± 0.08	6.60 ± 0.08
DETCA+fluvastatin	6.81 ± 0.06	5.81 ± 0.18	6.60 ± 0.13
DETCA+fluvastatin+L-NA	6.89 ± 0.08	5.85 ± 0.12	6.74 ± 0.09

In the present study it has been demonstrated that oxidative stress generated by inhibition of SOD impaired both endothelium-dependent relaxations (E_{max} values) and sensitivity (pD_2 values) to ACh, and attenuated the phenylephrine-induced contractile responses and sensitivity in isolated rings of rabbit carotid artery. However, incubation with DETCA did not affect contractile responses to serotonin in rings from carotid artery. On the other hand, our results showed that fluvastatin, a HMG-CoA reductase inhibitor, improved the reduced relaxant responses and sensitivity to ACh and restored the diminished contractile responses to PE without affecting the sensitivity to this agent.

In a number of studies it has been shown that treatment with DETCA caused severe impairment of NO-dependent relaxation which is consistent with our findings (14,15). It is well known that DETCA inactivates both intracellular and extracellular isoforms of Cu/Zn SOD (14). Thus, it is possible that inhibitor action of DETCA on relaxant responses may result from the accumulation of superoxide anion and/or inactivation of NO by superoxide anion (15).

In our study, the finding that fluvastatin treatment did not restore the ACh-induced relaxation in the absence of NO inhibition suggests the possible involvement of nitric oxide in this effect of this agent. Thus, considering the fact that DETCA-induced oxidative stress results from SOD inactivation, the finding that fluvastatin has superoxide anion scavenging activity against DNA damage (16) is consistent with our study. Moreover, in our previous study (17) and in results from Sumi et al. (18) in hypercholesterolemic conditions, fluvastatin has been shown to normalize the enhanced SOD activity in both erythrocytes and aortic tissue, and also to be involved in superoxide anion and eNOS.

In the present study, in the vitro experimental model of oxidative stress, either generated by inhibition of SOD or produced by accumulation of superoxide anion, treatment with DETCA blunted PE-induced contractions and sensitivity. In similar conditions to our experiment, DETCA has also been shown to reduce PE contractions in rat aorta (8). Several investigators have suggested that free radicals interfere with normal contractile function of vascular smooth muscle but the effects of these radicals on the contractile responses display inconsistent results in the literature (7-10). In a number of studies enhanced responsiveness to free radicals in mesenteric arteries of spontaneously hypertensive rats (19) and isolated rat aortic rings (9, 20) has been demonstrated. However, consistent with our findings, Wolin and Belloni have shown that xanthine oxidase-derived oxygen metabolites caused attenuation of norepinephrine-induced contractile tension but did not effect phenylephrine-induced contractions in strips of rabbit aorta (21). Moreover, Mizukawa and Okabe have reported that exogenous singlet oxygen depressed noradrenaline-induced contractions possibly *via* α-adrenoceptor dysfunction (7). Fluvastatin treatment fully reversed the blockade on maximum contractions but not in impaired sensitivity caused by DETCA. However, contractile responses in rings incubated together with L-NA and fluvastatin did not differ from responses in rings subjected to DETCA and fluvastatin. Therefore, this may suggest that the effect of fluvastatin-induced reversal of phenylephrine contraction is independent of the inhibition of NO. On the other hand, the finding that fluvastatin normalized the maximum PE contraction without affecting pD_2 values suggests that α_1-adrenoceptors may not play a role in this effect of fluvastatin. Moreover, the finding that incubation with DETCA significantly decreased both E_{max} and pD_2 values of PE raises the question of whether DETCA blocks the α-1 adrenergic receptor-mediated responses in the vessels or not. However, attenuated responses of serotonin under the same conditions have been reported in rat vessels (22). In this context, the ineffectiveness of DETCA on serotonin-induced contractions means that the differences between animal species regarding α_1-adrenergic receptor-mediated responses should be investigated.

In conclusion, the results of the present study showed that DETCA-induced oxidative stress blunted the contractile and relaxant responses to ACh and PE, and fluvastatin reversed these diminished responses in rabbit carotid artery. Further studies in order to elucidate the mechanism(s) of the oxidative stress-induced impairment of vascular responses are still to be carried out. In most studies while sophisticated techniques are being used for clarification of the role of the molecules/proteins and/or contractile elements under the mechanism of impaired vascular responses, the results of functional studies from organ chamber experiments still provide satisfactory results (20, 23).

In this context, it is worth noting that in spite of the fact that further studies are required to clarify the mechanism of the DETCA-induced impaired vascular responses, the results of these functional studies may also be of significance. Moreover, data from our experiments could contribute to an understanding of the additional beneficial effects of statins in addition to their effect of lowering cholesterol in patients with coronary heart disease.

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