MEDICAL CHANNEL

Original Article

ROLE OF PITAVASTATIN IN ENHANCING ENDOTHELIAL-DEPENDENT RELAXATION IN RAT AORTIC RING PREPARATIONS

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ABSTRACT

Introduction: Hypercholestrolemia-induced endothelial dysfunction is present in early stages of atherosclerosis. HMG-CoA reductase inhibitors improve endothelial dysfunction by limiting vasoconstriction.
Objective: To study effect of pitavastatin administration on vasoconstriction and vasodilation in isolated rat aortic rings.
Design: In-vitro pharmacological experiment.
Methods: Male albino rats were divided into Control group (n=8) and Pitavastatin treated group (n=8) given oral pitavastatin (0.4mg/kg/day) for 1 week. Graded dose-response-curves on rat aortic ring preparations were constructed to ascending doses of norepinephrine in control and pitavastatin treated rats. The relaxant effect of cumulative doses of acetylcholine on norepinephrine-induced submaximal contraction was studied with endothelium preserved and denuded.
Results: Isometric tension in rat aortic ring, produced by ascending concentrations of norepinephrine was significantly lower in pitavastatin treated than in control. In endothelium preserved preparations. acetylcholine produced significantly greater relaxation of epinephrine-induced contractions in the treated group. In endothelial denuded preparations, acetylcholine did not relax the tension at low doses, while increase in tension was observed at high doses.
Conclusion: Rats treated with pitavastatin show a significant improvement of endothelial reactivity as early as 1 week following therapy.

Keywords: Pitavastatin, aortic ring preparation, endothelial dysfunction, pleiotropic effects

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INTRODUCTION

The vascular endothelium in its normal quiescent state plays a crucial role in hemostasis and tissue perfusion by providing a smooth non-thrombotic surface and maintaining vessel tone[1]. A delicate balance exists between endothelium synthesized vasoconstrictors e.g. endothelin (ET-1), prostanoids; and vasodilators e.g. nitric oxide (NO), prostacyclin (PGI_2). The endothelium also modifies response to circulating vasoactive substances such as bradykinin and thrombin. Vascular

dysfunction or vascular activation produced in conditions like atherosclerosis, dyslipidemias, hypertension, diabetes etc target endothelium to tip the balance in favor of vasoconstricters[2]. The HMG-CoA reductase inhibitors are the most extensively used lipid lowering agents for prevention of atherosclerosis and cardiovascular events. They have been shown to enhance vasodilation and attenuate vasoconstrictor influences on the vessel wall[3] thereby improving tissue perfusion. In patients with hypercholesterolemia treatment with simvastatin 20mg/day for 4 weeks, significantly increased vasodilator response to acetylcholine (ACh) and improved forearm blood flow[4]. Research shows that statins can reduce endothelial synthesis of ET-1 by down-regulating prepro-endothelin mRNA level in endothelial cells through blocking RhoGTPase geranyl-geranylation[5]. The crucial effect of statins is exerted through their effect of NO levels. The possible mechanisms involved could be though increased eNOS production [6], increased NO activation[7] and decreased degradation through decreased production of reactive oxygen species[8]. Hence higher levels of NO diffuse across the endothelial lining into the smooth muscle layer resulting in vasodilation through cGMP pathway. Reduced bioavailabilty of NO due to endothelial dysfunction is considered to be the initial step in pathogenesis of atherosclerosis. Studies have found improvement in statin-induced endothelial functions even before a significant improvement in lipid levels[9]. Moreover this effect is not seen with non-statin lipid lowering therapy[10] suggesting that this might be a cholesterol independent or pleiotropic effect of statins. Pitavastatin is a recently introduced third generation statin with good lipid solubility and the highest bioavailability among the group. The level of pitavastatin in systemic circulation is higher than other lipid soluble statins[11]. Therefore pitavastatin is a suitable candidate for investigating peripheral effects.

The aim of this study was to assess the effect of pitavastatin administration on vasoconstrictive response to norepinephrine on rat aortic ring preparations and to evaluate the relaxant effect of acetylcholine on norepinephrine induced contraction in both endothelium intact and endothelial denuded rat aortic rings.

Impact of pitavastain on endothelial dysfunction is critical for assessment of improvement in tissue perfusion to myocardium and periphery. A direct vasodilator response could improve blood flow to ischemic tissues, stabilize atherosclerotic plaques and improve prognosis in patients with ischemic disease.

METHODOLOGY

The study was performed in accordance with the Jinnah Post-Graduate Medical Center Guidelines for use of experimental animals. It was approved by Animal Ethics Committee, approval number (F.1-2BMSI-E/012/JPMC, dated 15th Oct 2012).

Male Sprague-Dawley rats weighing 250 – 300 grams, aged 3 months were used.

The animals were housed in cages (4 animals per cage) with *ad libitum* access to food and water. On the day prior to the experiment the rats were fasted overnight with free access to water only.

Reagents:

Pitavastatin tablets (4 mg) Livalo Japan. Pitavastatin suspension (0.4mg/ml) was prepared in 10ml of distilled water for oral administration with a dose of 0.4mg/kg/day which is equivalent to the highest therapeutic dose in humans.

Norepinephrine (NE) injections (Norepine, Sterop). Ampoule contained 1mg/ml base. From this serial dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} M solutions were prepared

Acetylcholine powder (Sigma chemicals). A stock solution was prepared containing 10mg/ml ACh. From this serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}M solutions were prepared.

Kreb's Solution: Composition gm/L (mM): NaCl 5.54 (118), KCl 0.35 (4.7), $MgSO_4$, $7H_2O$ 0.29 (1.2), $CaCl_2$ 0.28 (2.5), KH_2PO_4 0.16 (1.17), $NaHCO_3$ 2.1 (20), Glucose 2.1

Aereation: 95% oxygen + 5% carbon dioxide

Temperature adjusted: 37°C

Animal groups: Rats were randomly allotted to one of the two groups.

Control Group (n =8) received 0.1ml of distilled water by oral tube for 1 week

P Group (n=8) received pitavastatin (0.4 mg/kg/day) suspension by oral feeding tube for 1 week

Procedure: Rats were killed by exsanguination. Chest was opened by a V shaped incision through the left and right lateral aspect of the rib cage starting from the midline till the under the arms. The heart and lungs were removed to expose the thoracic aorta. The distal end of the aorta where it passes through the diaphragm was cut and quickly and gently dissected up to the proximal end to yield a tube about 3-5 cms in length. The aorta was placed in a petri dish containing aereated Krebs solution. Using fine forceps, the adherent connective tissue and fat was removed. The aorta was cut into about 4 mm pieces. A ring was mounted horizontally between 2 triangular fine stainless steel wires passed through the lumen of the ring. The lower loop was fixed to the hook in the inner bath and the upper loop was connected to a force displacement transducer. The inner bath (volume 25ml) was filled with Krebs solution through which 95% O_2, 5% CO_2 was bubbled at a steady slow rate. The tissue was allowed to equilibrate under a tension of 1gm for 45 minutes washing every 10 minutes and readjusting the tension to 1 gm whenever needed. In endothelial denuded preparations the endothelium was removed by gently rotating fine forceps in the lumen of an aortic ring.

The contractile response of the aorta (force of contraction in gm) was recorded using an isometric transducer connected to data acquisition system (MacLab, ADInstruments).

1. Preconditioning of aortic ring: After 45 mins equilibration, the aorta was challenged with 10^{-6}M norepinephrine (25µL of 10^{-4}M solution) to assure good contractile condition of the preparation. It was left for 5 mins to reach the plateau. In preparations with intact endothelium, 10^{-5} M ACh (0.25 ml of 10^{-3}M solution) was added to the pre-contracted tissue for 5 min to test for endothelial integrity. If the vessel relaxed by at least 70%, it was used for further study. If the vessel ring failed to contract/relax, it was replaced by another ring from the same animal and the preconditioning steps repeated.
2. The tissue was washed again 3 times with Krebs solution and allowed to equilibrate for 30 minutes, washing at 10-minute intervals readjusting the tension to 0 g as needed.
3. A graded dose–response curve of norepinephrine was constructed starting with 10^{-8}M to $3X10^{-5}$M.
4. From this curve the concentration of norepinephrine that produced about 80% contractile response was repeated and left in bath for 5 mins. Acetylcholine was added in a cumulative manner (10^{-8} to 10^{-5} M) during the tonic contraction phase induced by norepinephrine.

Calculation: % relaxation = force of contraction by chosen NE dose – force of contraction after adding ACh dose / force of contraction by chosen NE dose X100

The same protocol was followed in both the pitavastatin treated and normal control groups with endothelium intact and denuded preparations.

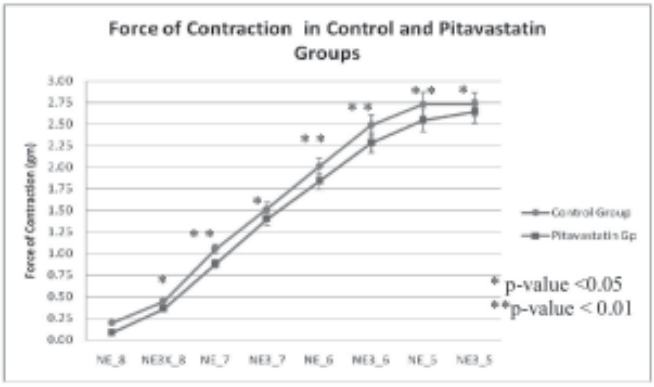
Statistical analysis: Values were expressed as mean ±SEM. Vasodilation induced by ACh was expressed as a percentage of relaxation of NE-induced contraction. Statistical evaluations of the data were carried out with unpaired Student's t-test for comparison of a single observation between control and pitavastatin treated rats. p- value<0.05 was considered statistically significant.

TABLE 1:
Isometric Tensions (gm) in Rat Aortic Rings (Endothelium Intact) Induced by Ascending Concentrations of Norepinephrine.

Final bath concentration (M) of NE	Control Group (n=8)	Pitavastatin Group (n=8)	p-value
1 X 10^{-8}	0.193 ± 0.136	0.081± 0.113	0.095
3 X 10^{-8}	0.443 ± 0.068	0.258± 0.082*	0.042
1 X 10^{-7}	1.052 ± 0.122	0.482± 0.064 * *	0.006
3 X 10^{-7}	1.52 ± 0.089	0.99± 0.114 *	0.028
1 X 10^{-6}	2.011 ± 0.131	1.237± 0.049 * *	0.007
3 X 10^{-6}	2.48 ± 0.099	1.778± 0.056 * *	0.000
1 X 10^{-5}	2.73 ± 0.057	1.895± 0.038 * *	0.000
3 X 10^{-5}	2.731 ± 0.054	1.943± 0.080 *	0.027

* p-value <0.05
**p-value < 0.01

FIGURE 1:
Isometric Tension (gm) in Rat Aortic Rings Induced by Different Concentrations of Norepinephrine



RESULTS

1. Effect of Norepinephrine on Rat Aortic Rings

The data below (table 1, figures 1 and 3) shows details of the effects of ascending concentrations of norepinephrine in control and pitavastain treated rat aortic ring preparations, with intact endothelium.

The bath concentration of 10^{-8}M, NE produced a mean force of contraction was not significantly different in control group Vs the pitavastatin group. The force of contraction produced by higher doses of norepinephrine in control group was significantly higher (p <0.05) than the mean force in the pitavastatin group.

2. Effect of Acetylcholine on Norepinephrine-Induced Contractions (Endothelium Intact)

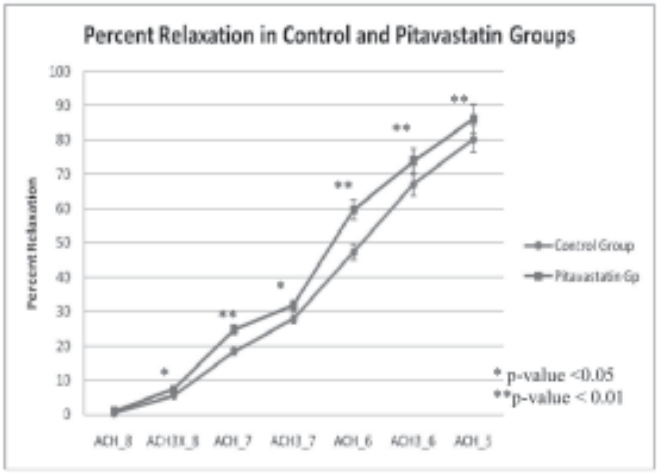
The data below (table 2, figures 2 and 4) shows details of the effects of ascending concentrations of acetylcholine in

TABLE 2
Acetylcholine-Induced Relaxation in Control and Pitavastatin Treated Rat Aortic Rings (Endothelium Intact)

Final bath Conc (M)	% relaxation			Force of contraction (gm)		
	Control (n=8)	Pitavastatin (n=8)	p-value	Control (n=8)	Pitavastatin (n=8)	p-value
NE $3X10^{-6}$				2.055	1.790	0.02
				± 0.123	± 0.079	
ACh $1X10^{-8}$	0.426	1.07	0.195	2.046	2.265 * *	0.001
	±1.077	± 0.808		± 0.126	± 0.071	
ACh $3X10^{-8}$	5.458	7.318 *	0.035	1.942	2.115 * *	0.006
	±1.906	± 1.082		± 0.118	± 0.094	
ACh $1X10^{-7}$	18.201	24.675 * *	0.001	1.678	1.725	0.298
	± 3.137	± 2.777		± 0.078	± 0.091	
ACh $3X10^{-7}$	27.768	31.807 *	0.014	1.483	1.562	0.055
	±2.222	± 3.304		± 0.088	± 0.058	
ACh $1X10^{-6}$	47.215	59.638 * *	0.000	1.085	0.925 *	0.020
	±2.575	± 5.950		± 0.089	± 0.143	
ACh $3X10^{-6}$	66.690	73.695 * *	0.003	0.681	0.601	0.109
	±3.216	± 4.00		± 0.097	± 0.088	
ACh $1X10^{-5}$	80.222	86.198 * *	0.001	0.406	0.311 *	0.012
	±2.268	± 3.163		± 0.054	± 0.073	

* p-value <0.05
**p-value < 0.01

FIGURE 2:
Acetylcholine-Induced Relaxation (%) in Control and Pitavastatin Treated Rat Aortic Rings (Endothelium Intact)



control and pitavastain treated rat aortic ring preparations, precontracted with norepinephrine, with intact endothelium. The $3X10^{-6}$M dose of norepinephrine produced approximately 80% of maximal contraction.

Mean relaxation produced by bath concentration of 10^{-8} M ACh was not significantly different (p=0.195) from the mean relaxation in pitavastatin group. The mean relaxation produced by higher doses of ACh in control group, was significantly less (p<0.05) than the mean relaxation in pitavastatin group.

3. Effect of Acetylcholine on Norepinephrine-Induced Contractions in Rat Aortic Rings (Endothelium Denuded)

The data below (table 3, figure 5) shows details of the effects of ascending concentrations of acetylcholine in control and pitavastain treated rat aortic ring preparations, precontracted with norepinephrine, with denuded endothelium. It was found that mean relaxation % produced by all doses of acetylcholine did not differ significantly in the control and pitavastatin treated rats after removal of endothelium. The higher doses i.e. ACh $3X10^{-6}$ and 10^{-5}M increased contraction of aorta by 8.33%±2.16 and 10.18%±2.72 respectively. However this was not significantly different than control group.

DISCUSSION

The endothelium is a major regulator of vascular homeostasis. It maintains vascular tone through synthesis and release of vasoactive contracting or relaxing factors in response to stimuli such as shear stress, pH, autacoids, cytokines etc. Upsetting this tightly regulated balance can produce endothelial dysfunction.

TABLE 3:
ACh-Induced Change in Force of Contraction in Control and Pitavastatin Treated Rat Aortic Rings (Endothelium Denuded)

Final bath Conc (M)	% relaxation			Force of contraction (gm)		
	Control (n=8)	Pitavastatin (n=8)	p-value	Control (n=8)	Pitavastatin (n=8)	p-value
NE $3X10^{-6}$				2.32 ±0.26	2.11±0.21	0.61
ACh $1X10^{-8}$	0.72	0.60		2.30	2.10	
	±1.84	± 1.41	0.77	± 0.267	± 0.19	0.39
ACh $3X10^{-8}$	0.23	0.39		2.31	2.10	
	±1.45	± 2.36	0.29	± 0.27	± 0.18	0.21
ACh $1X10^{-7}$	-0.74	-0.40		2.33	2.11	
	± 1.54	± 4.23	0.06	± 0.26	± 0.17	0.20
ACh $3X10^{-7}$	-4.18	-2.11		2.37	2.20	
	± 3.48	±3.65	0.97	± 0.25	± 0.17	0.29
ACh $1X10^{-6}$	-9.17	-4.96		2.43	2.32	
	± 5.05	±3.33	0.16	± 0.22	± 0.19	0.71
ACh $3X10^{-6}$	-12.95	-8.33		2.51	2.38	
	± 3.61	±2.16	0.07	± 0.25	± 0.19	0.24
ACh $1X10^{-5}$	-14.36	-10.18		2.55	2.42	
	± 3.41	± 2.72	0.071	± 0.27	± 0.22	0.45

- indicates increase in amplitude of contraction

FIGURE 3:
A Representative Tracing of Graded Dose-Response Curve of Norepinephrine in Rat Aortic Ring in Control Group

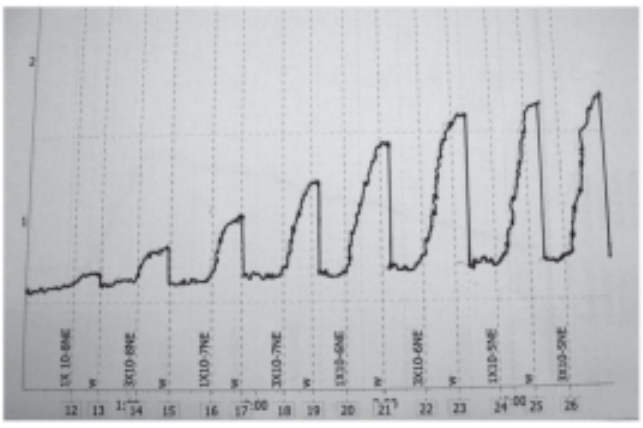
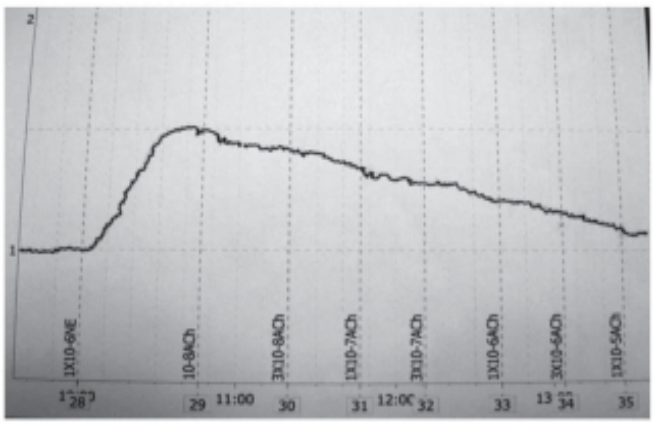


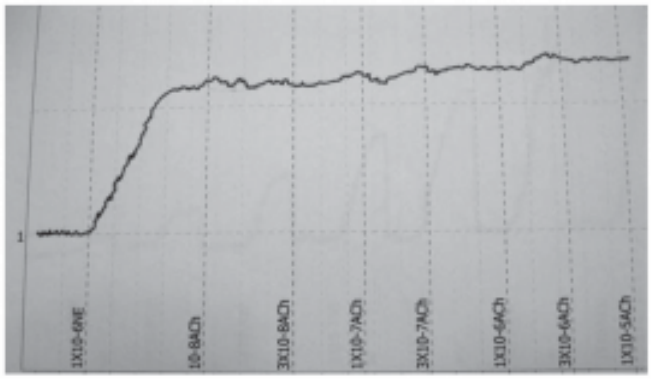
FIGURE 4:
Representative Tracing of Acetylcholine-Induced Relaxation in Rat Aortic Ring Pre-contracted With Norepinephrine (Endothelium Intact).



One method of functionally assessing endothelial dysfunction is by measuring endothelial-dependent dilation in vitro using isolated blood vessel or in vivo by measurement of flow mediated dilation in response to agonists. Previous studies have demonstrated a role of statins in improving endothelial dysfunction and modulating responses to vasoconstrictor and vasodilator substances. This response varies between hydrophilic and lipophilic statins with the hydrophilic statins showing no vasomotor effects[12]. In this study we aimed to assess the ability of pitavastatin, a novel, fully synthetic statin with good lipid solubility in modifying the response of a large conductance vessel to vasodilator and vasoconstrictor influences when administered in oral therapeutic doses.

The major findings of this study showed that the vasoconstrictor response to norepinephrine, added in

Figure 5:
Representative Tracing of Acetylcholine-Induced Relaxation in Rat Aortic Rings Pre-contracted with Norepinephrine with Endothelium Denuded.



ascending bolus doses, was significantly higher in control than in rats administered pitavastatin for 1 week. Our findings are in agreement with earlier studies by Ghaffari *et al.* (2011) who demonstrated that pravastatin pretreatment reduced both potency and maximal response to phenylephrine and thromboxane. This effect was abolished by endothelial denudation and inhibition of NOS[13]. Mraiche F *et al.* (2005) conducting isometric tension studies on rat thoracic aorta showed that simvastatin induced a concentration-dependent relaxation of tonic contractions mediated by endothelin-1 (10nM) and relaxation was also observed in rings contracted with NE (0.1 µM) and KCl (60mM). However in this study simvastatin was added in high concentration directly to the perfusing fluid[14]. Mechanism of action of simvastatin and atorvastin could be through inhibiting Ca^{2+} mobilization in vascular smooth muscles by blocking Ca^{2+} entry through the volage gated Ca^{2+} channels and release of Ca^{2+} from sarcoplasmic reticulum [15]. Mukai *et al.* (2003) found that at high concentrations (1-300µM) both cerivastatin and fluvastatin added to the perfusing fluid, directly relaxed blood vessels regardless of presence or absence of endothelium. Multiple mechanisms were found invoved in the dilator effect including augmentation of NO mediated relaxation through the Pl3-kinase/Akt pathway and Kv channels[16]. We did not find a direct relaxant effect of pitavastatin in endothelium denuded preparations, probably because oral administration of pitavastatin did not produce a high enough concentration in systemic circulation. Simvastatin was shown to produce vascular relaxation of both conductance and small vessels by acting on smooth muscles and endothelium. The relaxation involved release of both NO and vasodilators products from COX by a mechanism sensitive to O_2^- scavengers[17]. This relaxation involved the mevalonate and tyrosine kinase pathways. In vitro studies using cell cultures have found that pitavastatin induces NO production by vascular endothelial cells by inducing eNOS and protects against endothelial injury caused by TNF?.[18].

The present study also showed that pitavastatin was able to enhance the relaxant effect of a vasodilator substance – acetylcholine in large blood vessels by acting via an endothelium-dependent mechanism. In the presence of the intact endothelium the relaxation in the pitavastatin treated group was significantly higher (p=0.001) than control achieving 80.22±2.26% in control Vs 86.19±3.16% in pitavastatin group. In experiment where the endothelium was denuded, acetylcholine did not produce a significant relaxation of epinephrine induced contractions in both pitavastatin treated and control animals. However higher bath concentrations of ACh $3X10^{-6}$ M produced a mean increase in contraction of 12.95±3.61% in control and 8.33±2.16% in pitavastatin group. Also ACh in a bath concentration of 10^{-5} M produced a mean contraction of 14.36± 3.41% in control and 10.18±2.72% in pitavastatin group. However these values were not significantly different. Our study is in conformity with earlier report by Jorge *et al.* (2005) who demonstrated that the lipohilic statins administered by oral gavage for 15 days to hypercholestrolemic rabbits produced an endothelial dependent relaxation which was significantly greater in treated than in control animals [19].

CONCLUSION

This study showed that pitavastatin repressed norepinephrine induced contractions in rat aortic rings. It potentiated the vasodilator effect of acetylcholine which required the presence of an intact endothelium. This suggests that the relaxant action might be mediated via an endothelial derived factor. At the concentration achieved by systemic administration there was no direct relaxant effect of pitavastatin on vascular smooth muscles.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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