Bratisl Lek Listy 2008; 109 (11)
477 – 482

EXPERIMENTAL STUDY

Vascular reactivity in the experimental, simvastatin-treated diabetes with endothelial dysfunction

Samet Yalcin[1], Cüneyt Köksoy[2], Emre Ergül[1], Emine Demirel[3], Bülent Yalcin[4]

Ankara Atatürk Teaching and Research Hospital, Department of General Surgery, Ankara, Turkey.
dreergul@gmail.com

Abstract: *Background:* The aim of this study was to evaluate the effect of simvastatin therapy on general characteristics of diabetes and vascular reactivity in the 14 week-old streptozotosin-diabetic rats.
Methods: Twenty-four Sprague-Dawley male rats were divided into four groups as following: control, control-statin, diabetes and diabetes-statin.
Results: We observed that hyperglycemia and weight-loss observed in diabetic rats were partially treated with simvastatin, but were still different from the control group. After thapsigargin, the endothel-mediated acetylcholine releasing responses were decreased; the releasing response in diabetes-statin group differed from the diabetic group.
Discussion: Simvastatin treatment in diabetic rats, in addition to the treatment of diabetic dislipidemia, has also partially treated the endothel-mediated releasing response in diabetes. We observed that thapsigargin reduces the response of the aortic rings to the current substance *(Fig. 5, Ref. 15).* Full Text (Free, PDF) *www.bmj.sk.*
Key words: dislipidemia, simvastatin, streptozotocin-diabetes, thapsigargin, vascular reactivity.

Diabetes mellitus affects approximately 100 million people worldwide (1). Five to ten percent have type 1 (formerly known as insulin-dependent) and 90 % to 95 % have type 2 (non-insulin-dependent) diabetes mellitus. It is likely that the incidence of the type 2 diabetes will rise as a consequence of the lifestyle contributing to obesity (2). Cardiovascular physicians are encountering many of these patients because vascular diseases are the principal causes of death and disability in people with diabetes. The macrovascular manifestations include atherosclerosis and medial calcification. The microvascular consequences, retinopathy and nephropathy, are major causes of blindness and the end-stage renal failure. Physicians must be cognizant of the salient features of diabetic vascular disease in order to treat these patients effectively.

Statin therapy is a widely used treatment of hypercholesterolemia, reduces the risk of stroke and improves the cardiovascular functions. The statin family are competitive inhibitors of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, the rate limiting enzyme in the synthesis of cholesterol which converts HMG-CoA to mevalonate. Statins have anti-oxidant, anti-thrombotic and angiogenetic effects3. They also increase nitric oxide (NO) by up-regulating nitric oxide synthase (NOS) enzyme (3).

[1]Ankara Atatürk Teaching and Research Hospital, Department of General Surgery, [2]Ankara University School of Medicine, Department of General Surgery, [3]Ankara University School of Medicine, Departments of Pharmacology and Clinical Pharmacology, and [4]Ankara University School of Medicine, Department of Medical Oncology

Address for correspondence: Emre Ergul, Dr, Askaabat Cad. Eser Sitesi B Blok, 3. Giris Daire:11 06490 Bahcelievler, Ankara, Turkey.
Phone: +905056821500, Fax: +903122123414

Thapsigargin is a sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor. Thus, it empties the Ca^{2}-storage at the sarco/endoplasmic reticulum (SER) by inhibiting the active transportation of Ca^{2+} to the SER.

The aim of this study is to evaluate the effect of simvastatin therapy on general characteristics of diabetes and vascular reactivity in the 14 week-old streptozotocin (STZ) – diabetic rats.

Material and methods

The Ethics Committee of the Ankara University School of medicine approved the experimental procedures in this study. All of the guiding principles in the care and use of laboratory animals were strictly adhered throughout the entire study.

Twenty-four Sprague Dawley male rats, weighting between 200 and 250 g, were housed in a climate controlled (relative humidity of 30–70 % and temperature of 22 °C) animal-care facility, with a 12-hour light/dark cycle. The animals were provided with the standard rodent chow and water ad libitum.

The rats are divided into four groups as following: control (C), control-statin (CT), diabetes (D) and diabetes-statin (DT). Diabetes was achieved by 45 mg/kg single dose injection of STZ via tail-vein and the rats, whose blood glucose levels were 250 mg and above, were accepted as diabetic at the third day after the injection. Their plasma glucose levels and weights were measured every day. 8 weeks after the diabetes was achieved, 1 mg/kg simvastatin treatment was given via intraperitoneal route to both of the control-statin and diabetes-statin groups. During the 14 week-long experiment, without food or water restriction, all rats were taken care under the standard conditions. Plasma glucose,

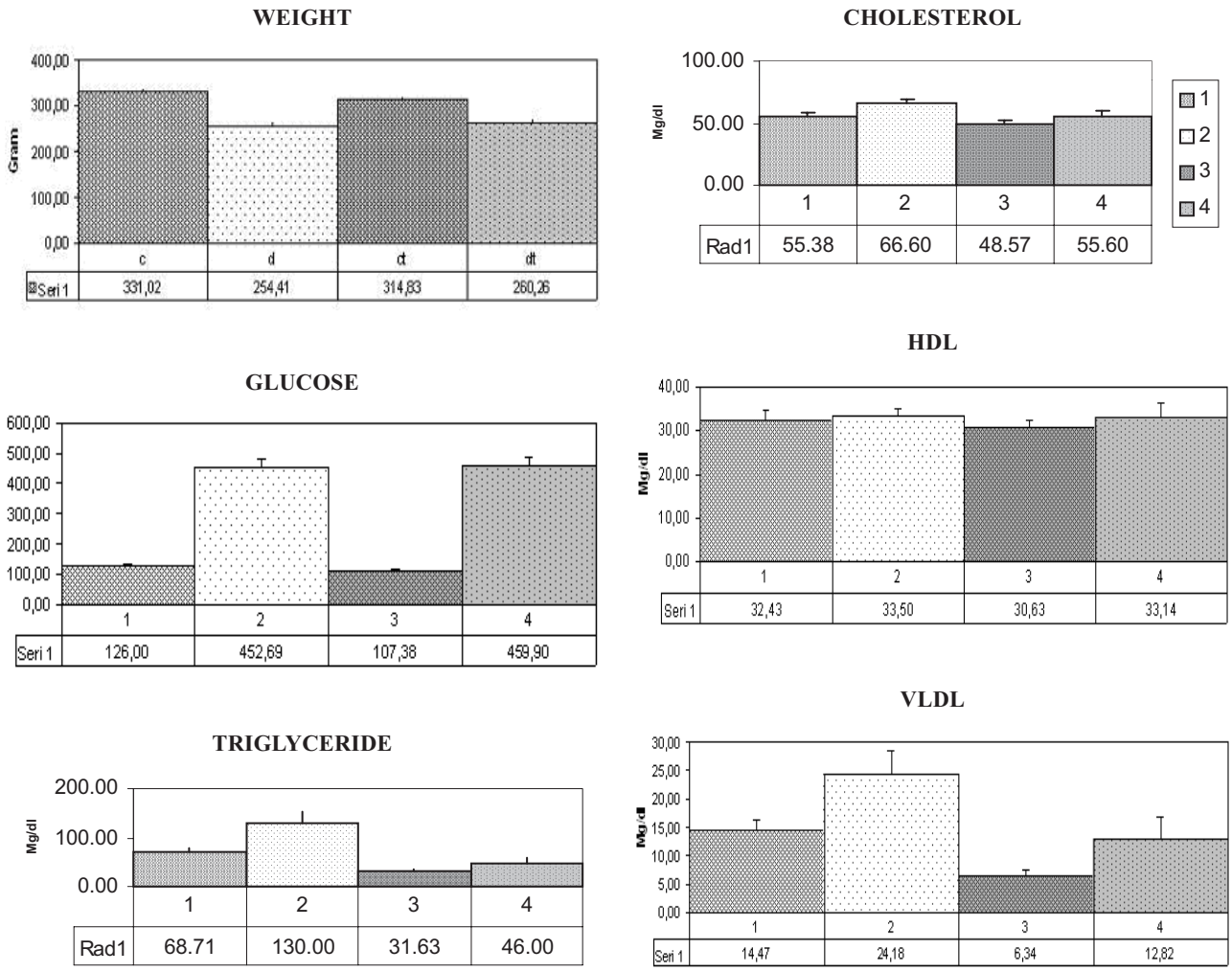


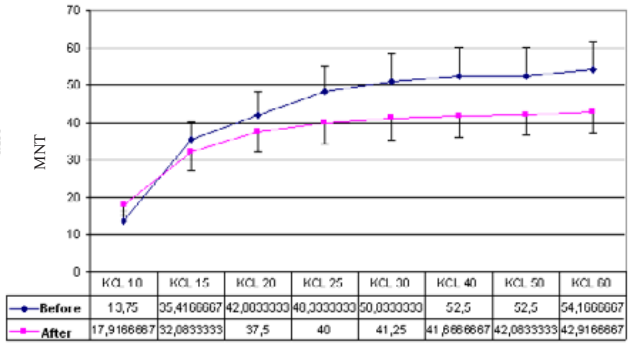
Fig. 1. Triacylglycerol, VLDL, HDL, plasma cholesterol, plasma glucose levels and weights of the groups at the end of the experiment. Group 1 (c) – control, Group 2 (d) – diabetic, Group 3 (ct) – control+Simvastatin, Group 4 (dt) – diabetic+Simvastatin.

total cholesterol, triglyceride and lipoprotein (VLDL, LDL, HDL) levels were measured fourteen weeks after the STZ injection.

Rats were anesthetized with an intraperitoneal injection of ketamine hydrochlorur (50 mg/kg) (Ketalar, Parke-Davis) and xylasine (10 mg/kg) (Rompun, Bayer) at the end of 14 weeks. The surgical field was shaved and prepared with 1 % antiseptic povidine-iodine solution and an incision was made along the thoracal midline. The thoracic aorta was quickly removed, and the isolated aorta cut into rings of 3 mm in length. The rings were suspended between two triangular-shaped stainless steel stirrups in a jacketed organ chamber filled with 20 ml modified Krebs-Henseleit solution. The modified Krebs-Henseleit solution was comprised of (in mM): 118 NaCl, 4.6 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 11.1 glucose, 27.2 $NaHCO_3$, 0.03 Na_2-ethylene-diamine-tetra-acetic acid (EGTA) and 1.8 $CaCl_2$. The chamber solution was kept at 36.5 °C and oxygenated with 95 % O_2 and 5 % CO_2. Organ bath was changed every ten minutes. The lower stirrup was anchored and the upper stirrup was attached to a force-displacement transducer (Nihon Kohden TB-652T, Tokyo, Japan) to record the isometric force. All rings were stretched to generate a resting tension of 2 g. After 40 minutes of incubation, an acetyl-choline induced relaxation was tested to evaluate the viability of the endothelium.

Four rings were taken from each rat's aorta (one with endothelium, three without endothelium). After taking a submaximal contraction with phenilephrine (10^{-7} M) at the ring with endothelium, an acetylcholine (10^{-9}–10^{-5} M) relaxation was gained. After a submaximal contraction with phenilephrine at the rings without endothelium, a Na-nitroprusside (10^{-11}–10^{-6} M) relaxation reaction was gained. A cumulative contraction with phenilephrine

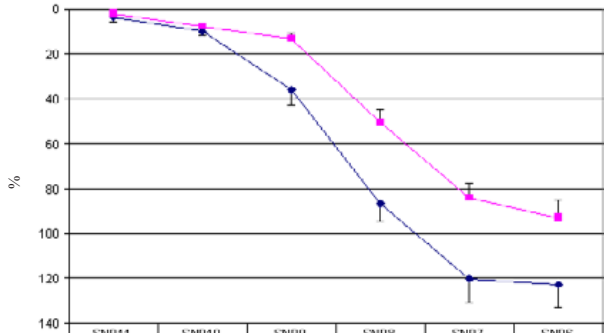
KCL response at diabetic group, before and after Thapsigargin



	KCL 10	KCL 15	KCL 20	KCL 25	KCL 30	KCL 40	KCL 50	KCL 60
Before	13,75	35,4166667	42,0833333	48,3333333	50,8333333	52,5	52,5	54,1666667
After	17,9166667	32,0833333	37,5	40	41,25	41,6666667	42,0833333	42,9166667

Fig. 2. KCL response in the diabetic group before and after thapsigargin. KCL has an increasing contraction response due to dose (10–60 µM) in all groups and there was no statistically significant difference in the same group before and after thapsigargin, except for the diabetic group ($p<0.05$).

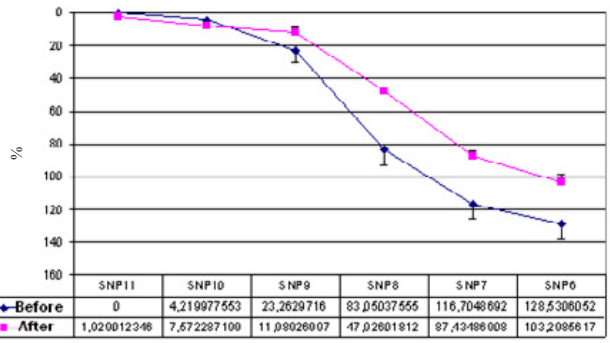
Na-Nitropussid response at diabetic group before and after Thapsigargine



	SNP11	SNP10	SNP9	SNP8	SNP7	SNP6
Before	3,52980353	9,980936937	36,04410083	86,88847384	120,0951705	122,7935832
After	2,104585976	7,698843423	13,21945153	50,33404783	84,09487555	93,05447288

Fig. 3. Na-Nitroprusside response in the diabetic group before and after thapsigargin. In the diabetic group Na-Nitroprusside had an increasing relaxation response due to dose (10^{-11}–10^{-6} M) ($p<0.05$).

Na-Nitropussid response at control-statin group before and after Thapsigargine



	SNP11	SNP10	SNP9	SNP8	SNP7	SNP6
Before	0	4,219977553	23,2629716	83,05037555	116,7048692	128,5306052
After	1,020012348	7,572297100	11,98026007	47,02601912	87,43498009	103,2086617

Fig. 4. Na-Nitroprusside response in the control-statin group before and after thapsigargin. In the control-statin group Na-Nitroprusside had an increasing relaxation response due to dose (10^{-11}–10^{-6} M) ($p<0.05$).

and a cumulative contraction with KCL were obtained at the other two rings separately. All the experiments were repeated after an addition of 10^{-6} M thapsigargin to the organ bath.

Data were expressed as mean±SD. Statistical evaluations of the vascular-response were obtained by Wilcoxon Signed Ranks for paired groups and Kruskal-Wallis Variance Analyze to compare four groups together. When a difference was found, a poly-variance analyze was performed. Data were analyzed with SPSS 11.5 for Windows.

Results

Hyperglycemia and weight-loss was partially observed in the simvastatin treated diabetic rats, but was still different from the control group. Simvastatin treatment had lowered the plasma triglyceride, very low density lipoprotein (VLDL) and cholesterol levels, which are the signs of dislipidemia, in diabetic rats compared to the control levels (Fig. 1). There was no statistically significant difference between all groups for the high density lipoprotein (HDL) levels.

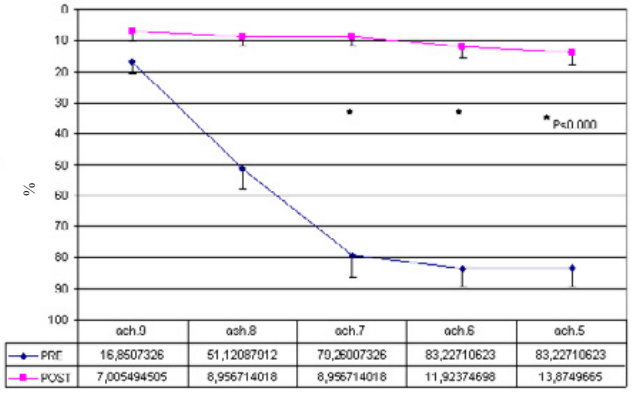
In the isolated aortic rings without endothelium, phenilephrine had an increasing contraction response due to dose in all groups and there was no statistically significant difference in the same group before and after thapsigargin, and also between the groups ($p>0.05$). KCL had an increasing contraction response due to dose (10–60 µM) in all groups and there was no statistically significant difference in the same group before and after thapsigargin, except for the diabetic group (Fig. 2). In the diabetic group, a stronger contraction response was obtained before thapsigargin then after. Na-Nitroprusside had an increasing relaxation response due to dose (10^{-11}–10^{-6} M) in all groups and there was no statistically significant difference in the same group before and after thapsigargin, except for the diabetic (Fig. 3) and control-statin (Fig. 4) groups.

In the isolated aortic ring of diabetic rats, after the pre-contraction performed with a single dose of Phenilephrine (10^{-7} molar) (before thapsigargin), it was observed that a decreasing response in the endothelial mediated responses with acetylcholine was partially treated with simvastatin. After the thapsigargin application in all groups, the endothel-mediated acetylcholine releasing responses were decreased (statistically significant) (Fig. 5), the releasing response in the treated group (diabetes-statin) differed more from the diabetic group.

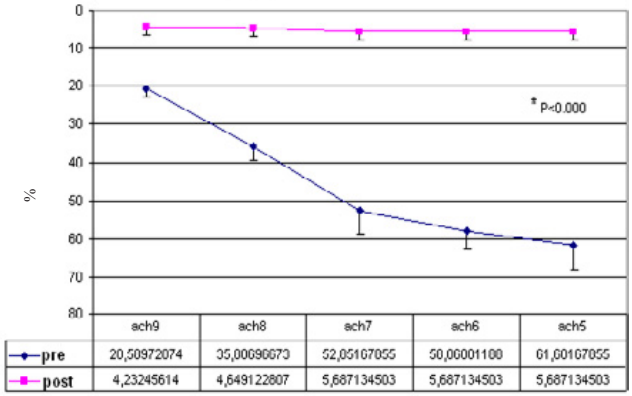
Discussion

Abnormalities in endothelial and vascular smooth muscle cell function, as well as propensity to thrombosis, contribute to athe-

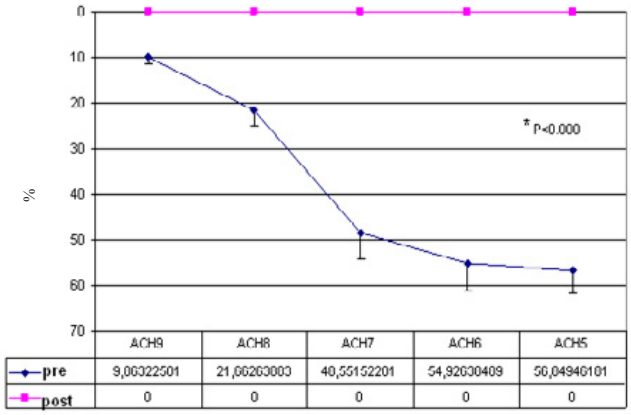
Acetylcholine response of Control group before and after Thapsigargine



Acetylcholine response of Diabetes group before and after Thapsigargine



Acetylcholine response of Control-statin group before and after Thapsigargine



Acetylcholine response of Diabetes-statin group before and after Thapsigargine

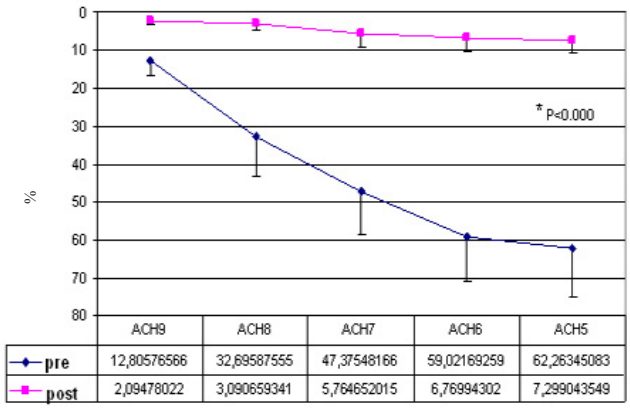


Fig. 5. Acetyl-choline responses of the groups with endothelium.

rosclerosis and its complications. Endothelial cells, because of their strategic anatomic position between the circulating blood and the vessel wall, regulate vascular function and structure. In the normal endothelial cells, biologically active substances are synthesized and released to maintain vascular homeostasis, ensuring an adequate blood flow and nutrient delivery while preventing thrombosis and leukocyte diapedesis. Among the important molecules synthesized by the endothelial cell is the NO, which is constitutively produced by endothelial NO synthase (eNOS). The bioavailability of NO represents a key marker in the vascular health. NO causes vasodilation by activating the guanylyl cyclase on subjacent vascular smooth muscle cells. In addition, NO protects the blood vessel from an endogenous injury by mediating molecular signals that prevent platelet and leukocyte interaction with the vascular wall and inhibit vascular smooth muscle cell proliferation and migration4. Endothelial dysfunction, as represented by an impaired endothelium-dependent, NO-mediated relaxation, occurs in cellular and experimental models of diabetes. Similarly, many, but not all, clinical studies have found that endothelium-dependent vasodilation is abnormal in patients with diabetes type 1 or type 2. Thus, decreased levels of NO in diabetes may underlie its atherogenic predisposition. Many of the metabolic derangements known to occur in diabetes, including hyperglycemia, excess free fatty acid liberation, and insulin resistance, mediate abnormalities in endothelial cell function by affecting the synthesis or degradation of NO. Thus, we performed the same procedures not only in aortic rings with endothelium but also without endothelium.

The intracellular glucose concentration of endothelial cells mirrors the extracellular environment. Experimental evidence supports the notion that hyperglycemia decreases endothelium-derived NO. When normal aortic rings are incubated in a hyperglycemic milieu, endothelium-dependent relaxation is impaired. Similarly, endothelium-dependent vasodilation is reduced in

healthy subjects during the hyperglycemic clamping. Hyperglycemia induces a series of cellular events that increase the production of reactive oxygen species (such as superoxide anion) that inactivate NO to form the peroxynitrite (2). Hyperglycemia may initiate this process by increasing the superoxide anion production via the mitochondrial electron transport chain. Superoxide anion then promotes a cascade of endothelial processes that engage increasing numbers of cellular elements to produce oxygen-derived free radicals. Activation of protein kinase-C (PKC) by glucose has been implicated in the regulation and activation of membrane-associated NAD-(P)H-dependent oxidases and subsequent production of the superoxide anion (5).

The concept that hyperglycemia-induced oxidative stress medi-supported by the observations that intra-arterial infusion of ascorbic acid, a water-soluble antioxidant capable of scavenging superoxide anion, restores endothelium-dependent vasodilation in healthy subjects exposed to a hyperglycemic clamp and in patients with type 1 or type 2 diabetes (2). Hyperglycemia also increases the production of the lipid second messenger diacylglycerol, which causes the membrane translocation and activation of protein kinase-C. Activation of PKC inhibits the activity of the phosphatidylinositol-3 kinase pathway, which results in a lower NO production.

We observed that hyperglycemia and weight-loss observed in diabetic rats are partially treated with simvastatin, but it was still different from the control group. Simvastatin treatment had lowered the plasma triglyceride, VLDL and cholesterol levels in diabetic rats. Circulating levels of free fatty acids were elevated in diabetes because of their excess liberation from adipose tissue and diminished uptake by skeletal muscle. Free fatty acids may impair endothelial function through several mechanisms, including increased production of oxygen-derived free radicals, activation of protein kinase-C, and exacerbation of dyslipidemia. Infusion of free fatty acids reduces endothelium-dependent vasodilation in animal models and in humans in vivo (6). Elevation of free fatty acid concentrations activate PKC and decrease insulin receptor substrate-1-associated phosphatidylinosital-3 kinase activity (7). These effects on signal transduction may decrease the NOS activity as discussed above. The liver responds to free fatty acid flux by increasing the very-low-density lipoprotein production and cholesteryl ester synthesis (8). This increased production of triglyceride-rich proteins and the diminished clearance by lipoprotein lipase results in hypertriglyceridemia, which is typically observed in diabetes. Elevated triglyceride concentrations lower HDL by promoting cholesterol transport from HDL to the very-low density lipoprotein (8). These abnormalities change LDL morphology, increasing the amount of the more atherogenic, small, dense LDL. Both hypertriglyceridemia and low HDL have been associated with the endothelial dysfunction (9).

In diabetes, endothelial cell dysfunction is characterized not only by a decreased NO but also by an increased synthesis of the vasoconstrictor prostanoids and endothelin (10). Endothelin may be particularly relevant to the pathophysiology of vascular disease in diabetes because endothelin promotes inflammation and causes vascular smooth muscle cell contraction and growth (11). Insulin increases endothelin-1 immunoreactivity in endothelial cells. Also, plasma endothelin-1 concentration increases after administration of insulin to healthy subjects and patients with type 2 diabetes mellitus (2).

Previous studies have suggested that the diabetic dyslipidemia induced impairment in vascular reactivity is largely due to alterations in coronary smooth muscle intracellular Ca^{2+} (Ca^{2+i}) regulation. These studies have shown that diabetic dyslipidemia significantly impairs Ca^{2+i} effluxes from the cell (11), increases basal Ca^{2+i} levels (11), and increases sarcoplasmic reticulum Ca^{2+i} buffering (12). Since it is currently thought that the increase in sarcoplasmic reticulum Ca^{2+i} buffering is a compensatory alteration due to the impairment in Ca^{2+} efflux and the rise in Ca^{2+i} levels, collectively these results suggest that pro-atherogenic factors present in the diabetic dyslipidemic state negatively impact the functional capacity of plasma membrane Ca^{2+} transporters (12).

Thapsigargin is a SERCA inhibitor. Thus, it empties the Ca^{2+}-storage at the SER by inhibiting the active transportation of Ca^{2+} to the SER. In the present study we observed that thapsigargin reduces the response of the aortic rings to the current substance. We also figured out that it increases the stage of ischemia.

We did not notice a study like this one. We evaluated the vascular (with and without endothelium) response to KCL, Na-Nitroprusside, and acetylcholine in control, control-statin, diabetic and diabetes-statin groups before and after thapsigargin administration.

In the isolated aortic ring of diabetic rats, after pre-contraction performed with a single dose of Phenilephrine (10^{-7} molar) (before thapsigargin), we observed that a decreasing response in endothelial releasing to acetylcholine was partially treated with simvastatin, as Pfaffman et al found (13). Different responses of diabetic aorta to the same substances have been found by different authors (13–15). Piper at al suggested that this variability is due to the duration of diabetes (15).

Our results have shown that simvastatin treatment in diabetic rats, in addition to treatment of diabetic dislipidemia, had also partially treated endothel-mediated releasing response in diabetes. Thapsigargin had a negative effect on vascular response not only in the group with endothelium but also without.

References

1. Amos AF, McCarty DJ, Zimmet P. The rising global burden of diabetes and its complications: estimates and projections to the year 2010. Diabet Med 1997; 14 (Suppl 5): 1—85.

2. Creager MA, Lüscher TF, Cosentino F, Beckman JA. Diabetes and Vascular Disease: Pathophysiology, Clinical Consequences, and Medical Therapy: Part I. Circulation 2003; 108; 1527—1532.

3. Endres M,Laufs U, Huang Z. Stroke Protection by the 3-Hydroxy-3-Methylglutarryl-CoA Reductase Inhibitors Mediated by Endothelial NO Synthase. Proc Natl Acad Sci 1998; 95: 8880—8885.

4. Collins T, Cybulsky MI. NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? J Clin Invest 2001; 107: 255—264.

5. Hink U, Li H, Mollnau H et al. Mechanisms underlying endothelial dysfunction in diabetes mellitus. Circulat Res 2001; 88: E14—E22.

6. Steinberg HO, Tarshoby M, Monestel R et al. Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. J Clin Invest 1997; 100: 1230—1239.

7. Dresner A, Laurent D, Marcucci M et al. Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. J Clin Invest 1999; 103: 253—259.

8. Sniderman AD, Scantlebury T, Cianflone K. Hypertriglyceridemic hyperapob: the unappreciated atherogenic dyslipoproteinemia in type 2 diabetes mellitus. Ann Intern Med 2001; 135: 447—459.

9. de Man FH, Weverling-Rijnsburger AW, van der Laarse A et al. Not acute but chronic hypertriglyceridemia is associated with impaired endothelium- dependent vasodilation: reversal after lipid-lowering therapy by atorvastatin. Arterioscler Thromb Vasc Biol 2000; 20: 744—750.

10. Luft FC. Proinflammatory effects of angiotensin II and endothelin: targets for progression of cardiovascular and renal diseases. Curr Opin Nephrol Hypertens 2002; 11: 59—66.

11. Hopfner RL, Gopalakrishnan V. Endothelin: emerging role in diabetic vascular complications. Diabetologia 1999; 42: 1383—1394.

12. Hill BJF, Price EM, Dixon JL, Sturek M. Increased calcium buffering in coronary smooth muscle cells from diabetic dyslipidemic pigs. Atherosclerosis 2003; 167: 15—23.

13. Pfaffman MA, Hilman R, Darby A. Contractilite and Relaxing Activity of Arterial Smooth Muscle from STZ-Diabetic Rats. Res Commun Pathol Pharmacol 1980; 30 (2): 283—299.

14. Sheykhzade M, Dalsgaard GT, Johansen T, Nyborg NB. The effect of long-term streptozotocin-induced diabetes on contractile and relaxation responses of coronary arteries: selective attenuation of CGRP-induced relaxations. Brit J Pharmacol 2000; 129: 1212—1218.

15. Piper GM, Peltier BA. Amelioration by L-Arginine of a Dysfunctional Arginine/ NO Pathway in Diabetic Endothelium. J Cardiovasc Pharmacol 1995; 25 (3): 397—403.

Received July 16, 2008.
Accepted September 20, 2008.