Effect of VULM 1457, an ACAT inhibitor, on serum lipid levels and on real time red blood cell flow in diabetic and non-diabetic hamsters fed high cholesterol-lipid diet

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Abstract. Acyl-coenzyme A: cholesterol O-acyltransferase (ACAT) catalyzes the formation of cholesterol/fatty acyl-coenzyme A esters. Accumulation of cholesterol esters leads to pathological changes connected with atherosclerosis.

We have evaluated effects of a newly synthesized ACAT inhibitor, 1-(2,6-diisopropyl-phenyl)-3-[4-(4'-nitrophenylthio)phenyl] urea (VULM 1457), on serum lipid (cholesterol and triglycerides) levels and velocity of red blood cells (RBC) in non-diabetic and diabetic hamsters fed on high cholesterol-lipid (HCHL) diet during 3 months. The VULM 1457 effects on the paw microcirculation were assessed using capillary microscopy by measuring (RBC) velocity *in vivo*.

Hamsters fed on HCHL diet became hypercholesterolemic with a dramatic increase in serum lipids accompanied with significantly decreased RBC velocity. Diabetic hamsters fed on HCHL diet had further increased serum lipids with reduction of RBC velocity. The VULM 1457 inhibitor lowered cholesterol levels in both non-diabetic and diabetic hamsters fed on HCHL diet. The greater VULM 1457 effect was shown in diabetic hamsters fed on HCHL diet where VULM 1457 expressed hypotriglycerides effects, too. An improved RBC velocity-pronounced effect was observed in diabetic hamsters fed on HCHL diet treated with VULM 1457. These results suggest that the ACAT inhibitor, VULM 1457, is a prospective hypolipidemic and anti-atherogenic drug which treats diabetes.

Key words: VULM 1457 — Streptozotocin — Cholesterol — Triglycerides — Videocapillaroscopy

Introduction

Acyl-coenzyme A: cholesterol O-acyltransferase (ACAT) catalyses the formation of cholesterol esters from cholesterol and the long-chain fatty acyl-coenzyme A. This enzyme plays an essential role in intracellular cholesterol storage, lipoprotein assembly, steroid hormone production and dietary cholesterol absorption (Kharbanda et al. 2005). Increased ACAT activity leads to the accumulation of cholesterol esters in the form of cytoplasmic lipid droplets within macrophages and smooth muscle cells, which in turn contribute to early lesions of human atherosclerotic plaques (Chang et al. 2001). Two ACAT genes have been identified in mammals: ACAT-1 and

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ACAT-2. ACAT-1 is expressed in all tissues, whereas ACAT-2 is highly expressed in the liver and the small intestine (Chang et al. 2006). After discovering of ACAT, two hypotheses have emerged. The first, in retrospective perhaps too simplistic: blocking cholesterol esterification in macrophages would diminish macrophage "foam cells" formation and thereby decrease atherosclerotic lesion development. The second hypothesis is that ACAT inhibition leads to decrease in hepatic and intestinal cholesterol ester formation, resulting in decrease in plasma levels of the atherogenic apolipoprotein B-containing lipoproteins (low-density lipoproteins (LDL) remnants derived from very low-density lipoprotein (VLDL) and chylomicrons) (Farese 2005).

Drugs inhibiting ACAT activity have been proved efficient in hypercholesterolemic and anti-atherogenic treatment (Kharbanda et al. 2005). Results of ACAT-1 inhibition showed toxic accumulation of unesterified cholesterol in the skin and brain in the setting of hypercholesterolemia (Accad

et al. 2000). This could be one reason why a recently tested non-specific ACAT inhibitor, pactimibe, failed in clinical tests (Nissen et al. 2006). A more optimistic finding indicates that inhibition of ACAT-2 results in a restricted capacity to absorb cholesterol and protection from diet-induced hypercholesterolemia and gallstone formations (Repa et al. 2004). The key question is whether the ACAT-2 activity is as important in the human liver as it was found in experiments on mice (Rudel and Shelness 2000).

One injection of a high dose of streptozotocin, a pro-diabetic chemical, induces β-cells necrosis within 4 h, rapidly followed by hyperglycemia. Streptozotocin was first described as an antibiotic with a broad spectrum, later as an antibiotic possessing oncolytic, oncogenic and diabetogenic properties (Bolzan and Bianchi 2002). Streptozotocin-induced diabetic animals, alike diabetic humans, suffer from micro-angiopathy, later developing into macro-angiopathy (Sotníková et al. 1999, 2006). Importantly, diabetic patients are affected by atherosclerosis earlier compared to healthy persons. Recently, the association between insulin resistance and endothelial dysfunction and the relationship between insulin resistance and ischemic heart disease has been reported (St-Pierre et al. 2005). Therefore, it seems that the curing procedure for diabetes should be considered in the context of dyslipidemia.

The most suitable method to determine earlier changes in blood flow is videocapillaroscopy. It is a non-invasive optical method to monitor real-time blood flow in micro-vessels (Cutulo et al. 2005). An important merit of the instrument is the possibility of conducting non-invasive investigations, i.e. without damage to the skin or vessels and not causing any pain or unpleasant sensations (Gurfinkel and Valery 2001). In the recent years, the interest in non-invasive study of capillary blood flow in cardiologic and diabetic patients has been raised mainly due to explicit microcirculation disorder in such pathological patients. The impaired endothelial function plays an important role in diabetes and the atherosclerotic process. Risk of developing diabetes increases levels of inflammatory cytokines and biochemical markers of endothelial dysfunction. In subjects with hypertension or diabetes, several abnormalities in the microvasculature have been reported (Keulen et al. 2002). Micro-vessel diameter and red blood cells (RBC) flow velocity changes during ischemia, reperfusion, as well as during vasoconstriction and vasodilatation, correlated with data obtained by the microscope (Vera et al. 2005). Despite the clinical effect of disease of microcirculation and macrocirculation, little information is available about the effect of blood-flow changes in the skin.

In this study, we investigated the effect of a novel non-specific ACAT inhibitor, VULM 1457 (Oremus et al. 2002), on the levels of serum lipids, including cholesterol and triglycerides, in non-diabetic and diabetic hamsters (Yamanouchi et al. 2000) fed on high cholesterol-lipid (HCHL) diet (Nistor et al. 1987). In addition, changes in RBC velocity were evaluated by videocapillaroscopy upon inhibitor treatment.

Materials and Methods

Chemicals and reagents

The VULM 1457 (1-(2,6-diisopropyl-phenyl)-3-[4-(4'-nitrophenylthio)phenyl] urea) was synthesized at Drug Research Institute, Inc., Modra, Slovakia. Streptozotocin, entelan, iron (III) chlorid, eozin (Merck, Bratislava, Slovakia), thiopental (Sandoz, Bratislava, Slovakia), hematoxylin, potassium iodide (Lachema, Czech Republic), oil red (Sigma, Germany).

Experimental animals and diet

A total of 50 male Syrian hamsters (Velaz, Praha, Czech Republic) of weight 85–107 g were individually housed in rodent cages. Animals were maintained in an environmentally controlled atmosphere (22–24°C, humidity 60%, 12/12 h light/dark cycle). The hamsters were fed on commercial rodent diet for 1 week before the treatment period to become acclimated to the facility.

After the acclimatization period, animals were assigned randomly into experimental groups and fed (10 g/day/animal) on experimental diets for 3 months. The control group (absolute control, $n = 10$) was fed on commercial standard chow for rodents. Group CHL (cholesterol-lipid, $n = 8$) was fed a HCHL diet (3% cholesterol and 15% butter) according to Nistor et al. (1987). Group CHL VULM 1457 (cholesterol-lipid with VULM 1457, $n = 9$) was fed on a HCHL diet including VULM 1457 (50 mg/kg/day/animal). This dosage was choosen according to previous experiments with hamsters in Drug Research Institute, Inc., Modra, Slovakia (unpublished data). In other atherosclerotic animal models (rats and rabbits), application of 50 mg/kg/day/animal VULM 1457 had potent effects, too (Vojtaššáková et al. 2006; Ťažká et al. 2002).

To induce diabetes, streptozotocin was injected intraperitoneally (i.p.) at a dose of 40 mg/kg of body weight on the first day of the experiment according to Yamanouchi et al. (2000). Hamsters with induced diabetes were divided into three groups. Group D (diabetes, $n = 7$) was fed on commercial standard chow for rodents, group D CHL (diabetes with cholesterol-lipid, $n = 6$) was fed on a HCHL diet and group D CHL VULM 1457 (diabetes with cholesterol-lipid with VULM 1457, $n = 7$) was fed on a HCHL diet including VULM 1457 (50 mg/kg/day/animal). Hamsters were kept freely on water, except for the diabetic groups, which were kept on 5% glucose solution during the first 24 h. This experiment

was carried out under the Animal Care, Use of Laboratory Animals, and with approval of the Animal Ethics Committee of the Faculty of Pharmacy, Comenius University.

Lipid measurements

Hamsters were anesthetized with an i.p. injection of thiopental (75 mg/kg of body weight). Blood samples from non-fasted animals were taken through a heart puncture, collected into heparinized tubes and centrifuged at $2500 \times g$ for 15 min to obtain the plasma. The levels of serum cholesterol, triglycerides and glucose were measured using commercial enzymatic kits CHOD-PAP (Cormay, Poland), GPO-PAP and GOD-PA (Pliva-Lachema, a.s., Czech Republic), respectively, and analyzed on bioanalyzer LISA 200 (Hycel, France).

Videocapillaroscopy

Left paws of thiopental-anesthesized animals were fixed and the capillaries were observed using a 10x objective (ZEISS Epiplan 10×/0.2 HD) on a capillaroscope controlled by a Capillaroscope software (Centre for Analysis of Substances, Joint Strock Company, Moscow 2001). For one sequence, twenty-five images of RBC were captured every second for a period of 10 s. To estimate RBC velocity, we measured the time of eight individual RBC that travelled a distance of 61–77 μm, which was set arbitrarily. The diameter of capillaries was equal in each experimental hamster (6–7 μm).

Statistical analysis

Differences between serum values of different experimental groups were determined using two-tailed Student's *t*-test for unpaired samples. All values are expressed as a mean ± SD and significance was set to $p < 0.05$.

Results

We evaluated three basic biochemical characteristics in serum (glucose, cholesterol and triglycerides) in non-diabetic and diabetic hamsters after 3 months of the experiment (Table 1).

As expected, glucose levels were significantly increased in all diabetic hamster groups compared to non-diabetic control. The highest glucose levels were exhibited in diabetic animals fed on HCHL diet compared to non-diabetic control ($p < 0.01$).

In all experimental groups fed on HCHL diet, the concentration of plasma cholesterol was increased. Total concentration of plasma cholesterol was 5.5-fold higher ($p < 0.001$) in CHL hamsters compared with those fed on commercial diet. D CHL hamsters had significantly higher cholesterol levels compared to non-diabetic hamsters fed on the same HCHL diet (1.5-fold; $p < 0.01$). VULM 1457 administration significantly ($p < 0.001$) decreased cholesterol levels in both non-diabetic (1.3-fold) and diabetic (4-fold) hamsters fed on the same diet. Therefore, we concluded that the hypo-cholesterolemic effect of this inhibitor was much greater in the diabetic group.

Similarly to cholesterol levels, levels of triglycerides were increased in CHL hamsters compared to the control group ($p < 0.001$). Comparable triglycerides levels were observed in non-diabetic and diabetic hamsters fed on the same HCHL diet, which is in contrast with the cholesterol levels (see above). VULM 1457 treatment in

Table 1. Serum glucose, total cholesterol and triglycerides levels in experimental groups

Group	Glucose (mmol/l)	Total cholesterol (mmol/l)	Triglycerides (mmol/l)
Control	7.81 ± 1.9	1.62 ± 0.2	0.88 ± 0.3
CHL	7.05 ± 1.9	8.82 ± 0.8 *** *vs.* control	4.21 ± 1.7 *** *vs.* control
CHL VULM 1457	8.24 ± 2.9	4.23 ± 0.7 *** *vs.* CHL	3.22 ± 1
D	10.32 ± 2.5 * *vs.* control	1.48 ± 0.2	1.19 ± 0.56
D CHL	11.69 ± 2.8 ** *vs.* control	13.47 ± 3.8 *** *vs.* control *** *vs.* CHL *** *vs.* D	4.68 ± 2.9 ** *vs.* control ** *vs.* D
D CHL VULM 1457	10.24 ± 2.8	3.35 ± 0.5 *** *vs.* D CHL * *vs.* CHL VULM 1457	1.75 ± 0.4 ** *vs.* D CHL * *vs.* CHL VULM 1457

CHL, cholesterol-lipid; VULM 1457, 1-(2,6-diisopropyl-phenyl)-3-[4-(4'-nitrophenylthio)phenyl] urea; D, diabetes; $^{*} p < 0.05$; $^{**} p < 0.01$; $^{***} p < 0.001$. Data are expressed as mean ± SD for 7–11 hamsters. * *vs.*
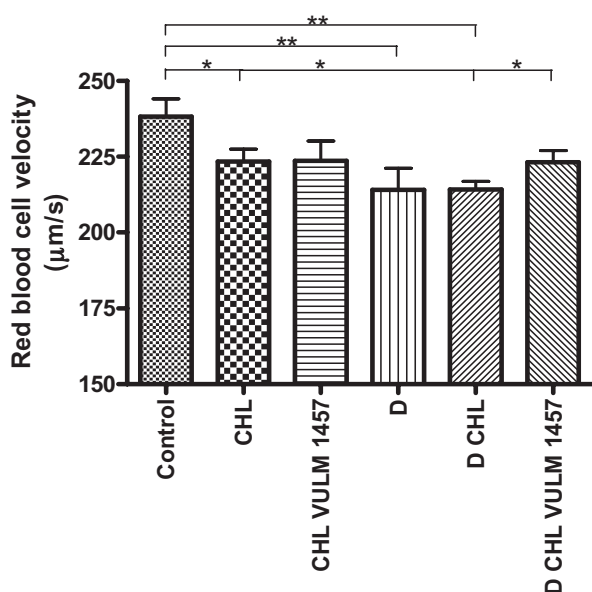
Figure 1. Analysis of RBC velocity in experimental groups. Data are expressed as mean ± SD for 6–9 hamsters. CHL, cholesterol-lipid; VULM 1457, 1-(2,6-diisopropyl-phenyl)-3-[4-(4'-nitrophenylthio)phenyl] urea; D, diabetes; * $p < 0.05$; ** $p < 0.01$.

diabetic hamsters fed on HCHL diet significantly reduced triglycerides levels compared to D CHL hamsters (2.7-fold, $p < 0.01$).

Next, we investigated changes in RBC as depicted in Figure 1. CHL hamsters had significantly decreased RBC velocity compared to the control group (6%, $p < 0.05$). Compared to the control group RBC, velocity reduction (10%, $p < 0.01$) was observed in diabetic and diabetic group fed on HCHL diet. D CHL hamsters had further reduced RBC velocity compared to non-diabetic hamsters fed on the same HCHL diet (4%, $p < 0.05$). After VULM 1457 administration, RBC velocity in diabetic hamsters fed on HCHL diet increased, compared to non-treated D CHL hamsters (4%, $p < 0.05$).

Discussion

In the present study, we used two previously established models of diabetes (Yamanouchi et al. 2000) and dyslipidemia (Nistor et al. 1987), to study interdependence of glucose and lipid metabolism. We found that streptozotocin-induced diabetic hamsters fed on HCHL diet had higher glucose, cholesterol and triglycerides levels than diabetic hamsters fed on standard diet. Additionally, in this group, RBC velocity was reduced more compared to non-diabetic hamsters and non-diabetic hamsters fed HCHL diet.

The consequences of interactions between diabetes and cholesterol-lipid metabolism are still not fully understood. There are 3 major components of dyslipidemia that occur in insulin resistance: increased triglycerides levels, decrease in HDL (high-density lipoproteins) and compositional changes in LDL (Lichtenstein and Schwab 2000). The data indicate that a HCHL diet leads to impairment of glucose tolerance. Impaired insulin binding and/or glucose transport has been previously related to changes in fatty acid composition of cellular membranes induced by dietary fat modification (Ťažká et al. 2000; Štefek et al. 2005). It is likely that common fundamental metabolic defects explain these abnormalities, because it is rare that they are found separately in insulin-resistant persons. Since the lipoprotein metabolism is interrelated with glucose metabolism, changes of lipid metabolism may explain all of the lipoprotein changes in dyslipidemia that characterize insulin resistance (Howard 1999).

Blood rheology is now receiving increasing attention as an important potential contributory factor of diabetic angiopathy. Hypertension, diabetes and hypercholesterolemia are known risk factors of development of the ischemic vascular disease. In dyslipidemic and diabetic conditions, RBC behaviour inside micro vessels remained controversial. RBC of male rats on a HCHL diet exhibited decreased ability to flow through capillaries when compared with those on a normal diet – a phenomenon which could not be explained by changes in geometric characteristics. The hematocrit deficit in the capillary with respect to the reservoir was greater in the HCHL diet fed rats than in the controlled ones. This was attributed to overall decrease in RBC deformability which, in turn, was related to increase in the membrane shear viscosity, the cytoplasm viscosity, the membrane shear modulus of elasticity and the membrane bending rigidity (Peddada et al. 1997). Elevated cholesterol levels, which impair RBC deformability and increase blood and plasma viscosity, may have an additional effect on hyperglycaemia in diabetes (Ercan et al. 2002).

Hori et al. (2004) described diabetes-induced dyslipidemia in streptozotocin-treated rats, which was caused in part by elevated intestinal ACAT activity. The non-specific ACAT inhibitor VULM 1457, a novel hypocholesterolemic and anti-atherosclerotic agent, is effective in prevention, development and regression of atherogenesis (Oremus et al. 2002). Oral administration of VULM 1457 to cholesterol-fed hamsters demonstrated a dose-related decrease in total cholesterol, LDL-cholesterol and VLDL-cholesterol in the plasma and the liver. The main mechanism of its hypolipidemic activity is associated with the inhibition of intestinal cholesterol absorption. The administration of VULM 1457 to cholesterol-fed rabbits induced regression of the pre-established atherosclerotic plaques of injured artery wall (Fáberová et al. 2002).

In our study, VULM 1457 showed hypolipidemic potency in both VULM 1457 treated, non-diabetic and diabetic hamsters fed on HCHL diet by reducing cholesterol levels. A greater VULM 1457 effect was shown in diabetic hamsters fed on HCHL diet, where VULM 1457 expressed hypotriglycerides effects, too. Interestingly, decreased lipid levels occured in VULM 1457-treated diabetic hamsters fed on HCHL diet, compared to VULM 1457-treated non-diabetic hamsters fed on the same diet. Furthermore, VULM 1457 considerably elevated RBC velocity in diabetic hamsters fed on HCHL diet. Burnett et al. (2005) observed that ACAT inhibitors have not only hypocholesterolemic, but also hypotriglyceride effects in cholesterol- and sucrose-fed rats, which is in accordance with our findings (St-Pierre et al. 2005). Moreover, ACAT inhibitors were shown to prevent formation of macrophage-derived foam cells in the arterial walls (Myiazaki et al. 2005). There is little information about hypolipidemic drugs and their microcirculation effect in preclinical as well as in clinical experiments. As one of the previous studies on hypolipidemic drugs shows, fluvastatin treatment improved microcirculation in patients with hyperlipidemia (Haak et al. 2001). We hypothetise that VULM 1457 may have a beneficial effect on microcirculation and RBC velocity. Up to the present, we have found no available information about measuring RBC velocity in diabetic and hyperlipidemic condition neither in hamsters nor in clinical studies.

Previous studies showed that intestinal ACAT activity was increased in diabetic rats and was brought to the normal levels by insulin treatment. This may suggest that insulin deficiency might lead to the elevated intestinal ACAT activity under the diabetic conditions (Lichtenstein and Schwab 2000; Hori et al. 2004). Based on our results, we speculate that decreasing the ACAT activity may contribute to improvement of diabetic conditions. We showed that VULM 1457 in diabetic hamsters fed on HCHL diet slightly but insignificantly decreased glucose levels compared to non-treated diabetic hamsters fed on HCHL diet.

Some of ACAT-inhibitors were shown to cause side effects, such as adrenotoxicity (Hori et al. 2004). However, this is not the case of the VULM 1457 inhibitor as it was well tolerated in rats and rabbits subjected to repeated oral administrations (Bezek et al. 2002).

In our study, VULM 1457 in diabetic and hyperlipidemic conditions had beneficial effects on the serum lipids levels as early as after 3-month treatment. We consider this ACAT inhibitor a drug with high potential to cure disregulated lipid metabolism during diabetes.

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