Available online at www.sciencedirect.com

SCIENCE DIRECT

Metabolism Clinical and Experimental 55 (2006) 794–802

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

Rapamycin, an mTOR inhibitor, disrupts triglyceride metabolism in guinea pigs

Dimple Aggarwal[a], Maria Luz Fernandez[a], Ghada A. Soliman[b,*]

[a]*Department of Nutritional Sciences, University of Connecticut, Storrs, CT 06269, USA*

[b]*Division of Metabolism, Endocrinology and Diabetes, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109, USA*

Received 9 November 2005; accepted 30 January 2006

Abstract

This study was designed to define some of the mechanisms by which rapamycin (RAPA), an mTOR inhibitor, induces hypertriglyceridemia when used as an immunosuppressive or antiproliferative agent and to determine whether low doses result in less undesirable side effects. Thirty male guinea pigs (n = 10 per group) were randomly assigned to control (no RAPA), low-RAPA (0.08 mg/d), or high-RAPA (0.85 mg/d) treatment for 3 weeks. Rapamycin treatment resulted in more than a 2-fold increase in plasma triglycerides (TG) ($P < .01$), whereas no differences were observed in plasma cholesterol between RAPA and control groups. Low-RAPA treatment resulted in lower concentrations of cholesterol in the aorta (28.6%) and lower hepatic acyl-CoA cholesteryl acyltransferase activity compared to control and high-RAPA groups ($P < .01$). In addition, acyl-CoA cholesteryl acyltransferase activity was positively correlated with aortic cholesterol ($r = 0.43$, $P < .05$). In contrast, aortic TG concentrations were higher in RAPA-treated guinea pigs than in control ($P < .01$). Very low density lipoprotein and low-density lipoprotein particles isolated from guinea pigs treated with RAPA were larger in size and contained more TG molecules than particles from control animals. Interestingly, plasma free fatty acids and fasting plasma glucose were 65% and 72% higher in the high-RAPA group than in control ($P < .01$). Tumor necrosis factor-α concentrations in the aorta were 3.6- and 10.4-fold higher in the low-RAPA and high-RAPA groups than in control guinea pigs ($P < .01$). These results suggest that RAPA interferes with TG metabolism by altering the insulin signaling pathway, inducing increased secretion of very low density lipoprotein and promoting deposition of TG in the aorta. Low RAPA was found to decrease cholesterol accumulation in tissue (liver and aorta) compared to high RAPA, suggesting that lower doses could be less detrimental to transplant patients.

1. Introduction

Rapamycin (RAPA), a lipophilic macrolide, has antifungal as well as potent antiproliferative and immunosuppressive effects. Because of its immunosuppressive properties, RAPA (also known as sirolimus) is a Food and Drug Administration–approved drug to prevent organ rejection in transplant patients. Furthermore, because of its antiproliferative action, RAPA-eluting stents are used in angioplasty and RAPA analogs hold promise as a novel anticancer agent [1]. Rapamycin unique effects are due to binding to the immunophilin FK506 binding protein (FKBP12). This binary complex of RAPA and FKBP12 interacts with RAPA binding domain (FRB) and thus inactivates a serine-threonine kinase termed the mammalian target of rapamycin or mTOR, which is known to control proteins that regulate mRNA translation initiation and G1 progression [2]. mTOR is an integrator of multiple signals receiving input from insulin, growth factors, amino acids, and energy to signal to the downstream targets and adjust cell growth and proliferation as well as metabolic homeostasis [3,4]. mTOR signaling is negatively regulated by tumor suppressor gene products Tuberous Sclerosis Complex (TSC-1 and TSC-2), PTEN, and LKB, and positively by proto-oncogene Ras homolog enriched in brain (Rheb), thus adding to the intricacy of mTOR regulation [5]. Furthermore, mTOR phosphorylates 2 well-characterized downstream targets, namely, ribosomal protein S6 kinases 1 and 2 (S6K-1 and S6K-2) and the eukaryotic initiation factor 4E (eIF-4E) binding protein (4E-BP1). Thus, RAPA, an mTOR inhibitor, leads to translational arrest by regulating S6K-1 and 4E-BP1. The immunosuppressive action of RAPA is due to inhibition of T-cell activation at a

* Corresponding author. Tel.: +1 734 763 6329; fax: +1 734 647 7979.
E-mail address: gsoliman@umich.edu (G.A. Soliman).

0026-0495/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2006.01.017

Table 1
Composition of experimental diets

Component	Control		Low RAPA		High RAPA	
	g/100 g	% Energy	g/100 g	% Energy	g/100g	% Energy
Protein[a]	22.3	23	22.3	23	22.3	23
L- Methionine	0.5		0.5		0.5	
Fat mix[b]	15.1	35	15.1	35	15.1	35
Corn starch/sucrose[c]	40.5	42	40.5	42	40.5	42
Mineral mixc	8.2	–	8.2	–	8.2	–
Vitamin mixc	1.1	–	1.1	–	1.1	–
Cellulose	10	–	10	–	10	–
Guar gum	2.5	–	2.5	–	2.5	–
Cholesterol	0.08	–	0.08	–	0.08	–
RAPA	0	–	0.0028	–	0.028	

[a] Mixture of soybean-casein (30:60). Fat mix contains olive oil–palm kernel oil–safflower oil (1:2:1.8), high in lauric and myristic acids.
[b] Corn starch–sucrose ratio (1:1.43).
[c] Mineral and vitamin mix adjusted to meet National Research Council requirements for guinea pigs.

later stage of the cell cycle, G1, and inhibition of S6K-1. However, this highly beneficial drug has an adverse effect of causing hyperlipidemias in renal, pancreatic, and liver transplant patients [6-9]. Rapamycin-associated dyslipidemia has been reported in 45% of liver transplant patients [10] and in about 40% of renal transplant patients [11]. In addition, phase II clinical trials described the occurrence of hypertriglyceridemia or hypercholesterolemia with administration of a RAPA analog (CCI-779) in the treatment of metastatic melanoma and glioblastoma multiforme [12]. Taken together, these observations indicate that hyperlipidemia is a considerable side effect of RAPA administration in both organ transplantation and as an antitumor treatment and may contribute to deteriorating the patients' condition. Understanding how the mTOR pathway regulates insulin signaling and triggers hyperlipidemia will help reduce risk of cardiovascular disease and insulin resistance (IR), 2 major medical problems of the United States.

The objective of this study was to define some of the parameters by which RAPA induces hypertriglyceridemia. A second objective was to evaluate the extent of side effects induced by low and high doses of RAPA. For this purpose, guinea pigs were used as the animal model because of their similarities to humans in terms of hepatic cholesterol and lipoprotein metabolism [13]. Previous studies done in our laboratory report the suitability of guinea pigs for evaluating various drugs affecting lipid metabolism [14-16].

2. Methods

2.1. Materials

Enzymatic cholesterol and TG kits, cholesterol oxidase, cholesterol esterase, and peroxidase were purchased from Roche-Diagnostics (Indianapolis, IN). Phospholipid (PL) and free cholesterol (FC) enzymatic kits were obtained from Wako Pure Chemical (Osaka, Japan). Quick-seal ultracentrifuge tubes were from Beckman (Palo Alto, CA). DL-Hydroxy-[3-^{14}C]methyl glutaryl coenzyme A (1.81 GBq/mmol), DL-[5-^{3}H]mevalonic acid (370 GBq/mmol), cholesteryl-[1,2,6,7-^{3}H]oleate (370 GBq/mmol), Aquasol, Liquiflor (toluene concentrate), and [^{14}C]cholesterol were purchased from DuPont NEN (Boston, MA). Oleoyl-[1-^{14}C]coenzyme A (1.8 GBq/mmol) and DL-3-hydroxy-3-methyl glutaryl coenzyme A were obtained from Amersham (Clearbrook, IL). Cholesteryl oleate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate, sodium fluoride, Triton, bovine serum albumin, and sucrose were obtained from Sigma (St Louis, MO). Glass silica gel plates were purchased from EM Science (Gibbstown, NJ).

2.2. Diets

Diets were prepared and pelleted by Research Diets (New Brunswick, NJ). Isocaloric diets were designed to meet all the nutritional requirements for guinea pigs. The 3 diets had identical composition except for the dose of tested drug (Table 1). The amount of drug incorporated in the diet of the 2 RAPA groups was calculated to provide 0.08 mg/d low RAPA, which mimics a 10 mg/d RAPA dose administered to renal transplant patients [7] or 0.85 mg/d (high RAPA), a dose comparable to that used in animal models [17,18] over a period of 3 weeks for each guinea pig. The amount of cholesterol in the diets was adjusted to be 0.08 g/100 g, an amount equivalent to 600 mg/d in the human diet [19].

2.3. Animals

To identify the metabolic pathway(s) leading to RAPA-mediated hyperlipidemia, 30 male guinea pigs (Harlan Sprague-Dawley, Hills, CT), weighing about 450 g, were randomly assigned to either a control, low dose of RAPA (low RAPA), or high dose of RAPA (high RAPA) for 3 weeks. All guinea pigs were fed a weighed amount of their respective test diet everyday, and the leftover was weighed the next morning. Two animals were housed per metal cage in a light cycle room (light from 7:00 AM to 7:00 PM) and had free access to water. Overnight fasted guinea pigs were sacrificed by heart puncture after

isoflurane anesthesia. Blood, livers, and aortas were harvested for analysis and were stored at −80°C for further analysis. All animal experiments were conducted in accordance with US Public Health Service/US Department of Agriculture guidelines. Experimental protocols were approved by the University of Connecticut Institutional Care and Use Committee.

2.4. Aortic tissue collection

After sacrificing, the heart and aorta were removed and the aorta was divided into 2 sections. The ascending aorta, aortic arch, and the upper portion of the descending aorta were removed and stored in phosphate buffered saline (PBS) at 4°C until homogenization could be performed for analysis of protein concentrations of tumor necrosis factor α (TNF-α). A section of thoracic aorta was stored in formalin at 4°C for measurement of aortic lipid concentration.

2.5. Lipoprotein isolation

Plasma samples were collected from blood obtained by heart puncture from guinea pigs under anesthesia. A preservation cocktail of aprotonin, phenyl methyl sulfonyl fluoride, and sodium azide was added to plasma samples to minimize changes in lipoprotein composition during isolation. Plasma was aliquoted for plasma lipid analysis, lipoprotein isolation, plasma free fatty acids (FFAs), and plasma insulin.

Lipoproteins were isolated by sequential ultracentrifugation in an LE-80K ultracentrifuge (Beckman Instruments, Palo Alto, CA). Very low density lipoprotein was isolated at d = 1.006 kg/L at 125 000 × g at 15°C for 19 hours in a Ti-50 rotor (Beckman). Low-density lipoprotein (LDL) was isolated at d = 1.019 to 1.09 kg/L in quick-seal tubes at 15°C for 3 hours at 200 000 × g in a vertical Ti-65 rotor [20]. Low-density lipoprotein samples were dialyzed in 0.9 g/L sodium chloride–0.1 g/L EDTA, pH 7.2, for 12 hours and stored at 4°C for further analysis.

2.6. Plasma, hepatic, and aortic lipids

Plasma samples were analyzed for cholesterol and TG by enzymatic methods [21]. Hepatic total and FC and TG were determined according Carr et al [22] after extraction of hepatic lipids with chloroform-methanol (2:1). Cholesteryl ester (CE) concentrations were calculated by subtracting free from total cholesterol. The section of thoracic aorta saved in formalin was cleaned thoroughly of any excess tissue and fat. Aortic cholesterol concentrations were analyzed as previously reported [23]. From the cleaned tissue, aortic lipids were extracted from approximately 0.05 g of aortic tissue arch using 10 mL of chloroform-methanol (2:1) overnight at room temperature. The extraction solution was mixed with acidified water to separate the solution into 2 phases, which was then filtered by gravity filtration and the lower phase extracted with a separatory funnel. An aliquot of 200 μL was then evaporated completely and reconstituted with 200 μL ethanol for enzymatic determination of total cholesterol. Another 2 mL was taken from the extract for TG determination using the enzymatic method.

2.7. Lipoprotein characterization

Very low density lipoprotein and LDL composition was calculated by determining free and esterified cholesterol [21], protein by a modified Lowry method [24], and TG and PLs by enzymatic kits. Very low density lipoprotein apolipoprotein B (apo B) was selectively precipitated with isopropanol [25]. The number of constituent molecules of LDL was calculated on the basis of one apo B per particle with a molecular mass of 412 000 kd [20]. The molecular weights were 885.4, 386.6, 645, and 734 for TG, FC and CE, and PLs, respectively. LDL diameters were calculated according to Van Heek and Zilversmit [26] as previously reported [16]. High-density lipoprotein (HDL) cholesterol was determined by a modified procedure, which consisted of using 2 mol/L $MgCl_2$ for precipitation of apo B–containing lipoproteins [14].

2.8. Hepatic microsome isolation

Hepatic microsomal fraction was isolated by two 25-minute centrifugations at 10 000 × g (JA-20 rotor, J221) followed by ultracentrifugation at 100 000 × g in a Ti-50 rotor at 4°C for 1 hour [15]. Microsomes were resuspended in the homogenization buffer and centrifuged for an additional hour at 100 000 × g. After centrifugation, microsomal pellets were homogenized and stored at −70°C.

2.9. Hepatic acyl-CoA cholesteryl acyltransferase activity

Hepatic acyl-CoA cholesteryl acyltransferase (ACAT) (EC 2.3.1.26) activity was measured by the incorporation of [^{14}C]oleoyl CoA in CE in hepatic microsomes by preincubating 0.8 to 1 mg of microsomal protein per assay with 84 g/L albumin and buffer for microsomal isolation [27]. Recoveries of [^{3}H]cholesteryl oleate were about 90%.

2.10. Plasma FFA concentration

Free fatty acids were determined from plasma by using acyl-CoA synthetase- and acyl-CoA oxidase. Briefly, 50 μL of plasma was treated with acyl-CoA synthetase in the presence of ATP, magnesium cations, and CoA to form acyl-CoA as well as AMP and pyrophosphate. Afterwards, acyl-CoA was oxidized by acyl-CoA oxidase to produce hydrogen peroxide which, in the presence of added peroxidase, allows the oxidative condensation to form a purple-colored adduct, which was read by the spectrophotometer at 550 nm.

2.11. Plasma glucose, insulin, and IR

Plasma glucose was determined enzymatically [28]. Briefly, 3 mL of working solution was added to 0.20 mL of sample and mixed, transferred to cuvettes, incubated at

Table 2
Total cholesterol, LDL-C, HDL-C, and TGs of guinea pigs treated with 0 (control), low dose, and high dose of RAPA*

Plasma lipids	Control (10) (mg/dL)	Low dose (10) (mg/dL)	High dose (10) (mg/dL)
TC	95.7 ± 14.7	83.8 ± 16.6	84.1 ± 43.3
VLDL-C	6.5 ± 1.1	7.8 ± 3.6	7.6 ± 4.3
LDL-C	78.4 ± 14.3	65.8 ± 17.2	68.3 ± 45.4
HDL-C	10.8 ± 3.3	10.2 ± 4.2	8.5 ± 2.6
TG	24.5 ± 5.9[a]	55.2 ± 31.0[b]	56.9 ± 29.9[b]

* Values are mean ± SD for the number of guinea pigs indicated in parentheses. Values with different superscripts are significantly different as analyzed by 1-way ANOVA and LSD as post hoc test ($P < .01$). Weight gain was used as a covariate.

37°C for 5 minutes, and then read at 505 nm on a DU-640 UV spectrophotometer (Beckman Coulter, Fullerton, CA). To measure plasma insulin, we used a Rat/Mouse Insulin ELISA Kit by Linco Research (St Charles, MO), which was found to cross-react with guinea pig. Homeostatic model assessment (HOMA) [29] was used to calculate IR according to the following equation: HOMA IR = fasting insulin (μU/mL) × fasting glucose (mmol/L) ÷ 22.5.

2.12. Aortic TNF-α

The section of ascending aorta, aortic arch, and the upper portion of the descending aorta stored in PBS were processed for cytokine determination. Aortas were thoroughly cleaned of excessive tissue and fat to avoid erroneous determinations of TNF-α concentrations in the aortic tissue. The cleaned portion of the aorta was rinsed with PBS and then cut into small segments and homogenized in 1 mL of lysis buffer in a rotor-stator (VirTis, Gardiner, NY) on ice for 60 to 90 seconds to ensure complete disruption of the aorta, then briefly in a Potter-Elvehjem homogenizer in another 2 mL of lysis buffer on ice for another 60 seconds. The homogenized tissue was centrifuged at 400 × g for 10 minutes at 4°C, and the supernatant collected and stored at −80°C for measurement of the aortic TNF-α [30]. Tumor necrosis factor-α was determined in duplicate using mouse ELISA kit from Endogen (Rockford, IL). The sensitivity of TNF-α was less than 10 pg/mL and the intra-assay coefficient of variation was 8.5%. Absorbances were read in a Titertek microplate reader (BioRad, Hercules, CA).

Table 3
Very low density lipoprotein number of CE, FC, PL, and TG molecules, and VLDL size of guinea pigs treated with 0 (control), low dose, and high dose of RAPA*

VLDL	Control (9)	Low dose (8)	High dose (9)
Number of molecules			
CE	117 ± 105	272 ± 246	459 ± 612
FC	2033 ± 810	2964 ± 1399	3097 ± 2348
PL	1416 ± 530	2445 ± 1209	2051 ± 1443
TG	2246 ± 1041[a]	13455 ±6187[b]	7877 ± 6068[b]
Size (nm)	18 ± 1.0[a]	63 ± 2[b]	47 ± 2[b]

* Values are mean ± SD for the number of guinea pigs indicated in parentheses. Values with different superscripts are significantly different as analyzed by one way ANOVA and LSD as post hoc test ($P < .01$). Weight gain was used as a covariate.

Table 4
Distribution of cholesterol in LDL fractions, LDL size, and LDL number of CE, FC, PL, and TG molecules*

LDL	Control (n = 9)	Low dose (n = 8)	High dose (n = 9)
Number of molecules			
CE	1728 ± 648	1793 ± 1048	2205 ± 1234
FC	626 ± 395	240 ± 249	514 ± 282
PL	427 ± 142	537 ± 345	703 ± 435
TG	105 ± 55[a]	147 ± 67[b]	170 ± 41[b]
LDL-1 (mg/dL)	46.1 ± 7.8	39.1 ± 13.1	41.6 ± 16.5
LDL-2 (mg/dL)	15.3 ± 3.9	12.1 ± 4.0	17.7 ± 17.5
LDL-3 (mg/dL)	11.8 ± 4.0[a]	4.8 ± 2.6[b]	5.3 ± 3.1[b]
LDL size (nm)	25.83 ± 0.31[a]	26.16 ± 0.49[ab]	26.30 ± 0.36[b]

* Values are mean ± SD for the number of guinea pigs indicated in parentheses. Values with different superscripts are significantly different as analyzed by 1-way ANOVA and LSD as post hoc test ($P < .001$).

2.13. Statistical analysis

One-way analysis of variance (ANOVA) (SSPS for Windows version 12, SPSS, Chicago, Ill) was used to evaluate significant differences among groups in regard to plasma, hepatic and aortic lipids, lipoprotein composition, FFA, ACAT activity, glucose, insulin, and TNF-α. The least significant difference (LSD) post hoc test was used to evaluate the differences among groups. Data are presented as the mean ± SD. Differences were considered significant at $P < .05$. Because of the differences in weight gain among groups, weight gain was used as a covariate in the determination of the different variables in this study.

3. Results

Guinea pigs treated with the higher dose of RAPA for 3 weeks had significantly lower ($P < .01$) weight gain than the other 2 groups. Weight gains over the period of 3 weeks

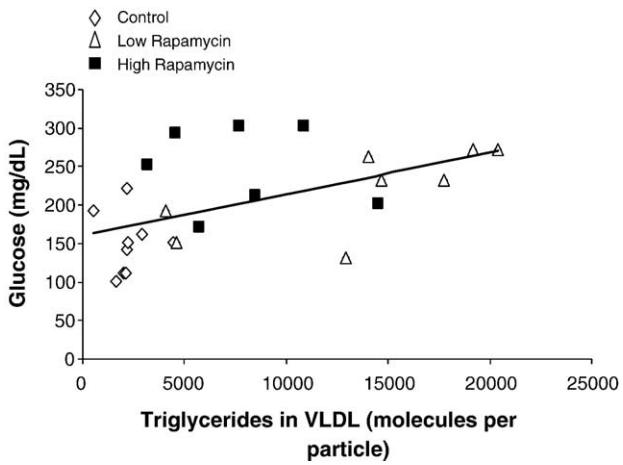

Fig. 1. Correlation between plasma glucose and TGs in VLDL ($r = 0.572$, $P < .01$) of guinea pigs treated with 0 (◇, control), low (Δ), or high (■) doses of rapamycin.

Table 5
Free fatty acids, plasma glucose, insulin, and calculated IR of guinea pigs treated with 0 (control), low dose, or high dose of RAPA*

	Control	Low dose	High dose
FFA (meq/L)	0.47 ± 0.13[a]	0.64 ± 0.13[ab]	0.78 ± 0.30[b]
Glucose (mg/dL)	148.5 ± 37.5[a]	211.1 ± 54.1[b]	255.0 ± 53.4[b]
Insulin (μU/L)	0.46 ± 0.08	0.54 ± 0.15	0.57 ± 0.29
Calculated IR	4.77 ± 1.00[a]	8.12 ± 3.57[b]	10.22 ± 5.60[b]

* Values are mean ± SD for 10 guinea pigs per group. Values with different superscripts are significantly different as analyzed by 1-way ANOVA and LSD as post hoc test ($P < .01$). Weight gain was used as a covariate.

were 40.7 ± 10.9, 38.7 ± 6.4, and 9.5 ± 9.1 g for guinea pigs treated with 0, low-, or high dose of RAPA, respectively. However, the amount of food consumed by the control (30 ± 5 g/d), low-dose (32 ± 4), or high-dose groups (28 ± 5 g/d) was not different.

3.1. Plasma lipids and composition of lipoproteins

The 2 doses of RAPA tested did not lead to any changes in plasma TC or cholesterol carried by any of the lipoproteins (ie, VLDL, LDL, or HDL) as compared to the control group (Table 2). However, there was more than a 2-fold increase in plasma TG values in both the RAPA groups as compared to the control ($P < .001$).

The composition of the VLDL particle was modified by RAPA intake as indicated by the larger size of VLDL isolated from RAPA-treated guinea pigs. The increase in size was associated with the number of TG molecules present in the VLDL particle, which contained 83.3% and 71.4% more TG molecules in the low-dose and high-dose groups, respectively, compared with VLDL isolated from control guinea pigs (Table 3). There were no significant differences in the number of CE, FC, or PL in VLDL isolated from the 3 treatment groups.

Similarly, the LDL particle isolated from the RAPA-treated guinea pigs had 28.1% and 38.2% higher number of TG molecules compared to the control animals (Table 4). There were no differences among groups in the number of CE, FC, or PL molecules. In addition, LDL particle size was also significantly ($P < .001$) larger in the 2 drug groups as compared to their control counterparts. In agreement with this observation, the concentration of LDL-3, the smallest LDL-subfraction, was lower in the RAPA-treated guinea pigs than in control animals (Table 4). A positive correlation was found ($r = 0.572$, $P < .01$) between the TG levels carried by the VLDL particles and the glucose content in the plasma (Fig. 1).

Table 6
Hepatic TC, FC, CE, and TG of guinea pigs treated with 0 (control) low dose, and high dose of RAPA*

	Control	Low dose	High dose
Hepatic lipids			
TC (mg/g)	2.08 ± 0.23	2.00 ± 0.17	2.24 ± 0.46
FC (mg/g)	1.43 ± 0.26[a]	1.39 ± 0.41[a]	1.83 ± 0.56[b]
CE (mg/g)	0.65 ± 0.18	0.61 ± 0.32	0.41 ± 0.40
TG (mg/g)	4.62 ± 2.85	2.66 ± 0.74	4.27 ± 1.48
ACAT activity (pmol/min · mg)	7.12 ± 3.67[a]	3.32 ± 1.97[b]	4.33 ± 3.34[ab]

* Values are mean ± SD for 10 guinea pigs per group. Values with different superscripts are significantly different as analyzed by 1-way ANOVA and LSD as post hoc test ($P < .01$). Weight gain was used as a covariate.

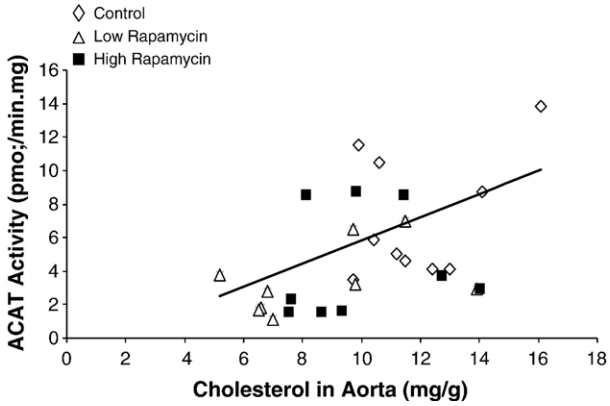

Fig. 2. Correlation between hepatic ACAT activity and cholesterol in aorta ($r = 0.43$, $P < .05$) of guinea pigs treated with 0 (◇, control), low (Δ), or high (■) doses of rapamycin.

3.2. Free fatty acids, insulin, glucose, and IR

Significantly more (65.9%) fatty acids were released in the high-RAPA group than in the control group (Table 5). Concentrations of FFAs in the plasma of low-RAPA group were higher than those of the control group, but the difference failed to reach statistical significance. Fasting plasma glucose values were significantly increased in low-RAPA (211.1 ± 54.1 mg/dL) and high-RAPA (255.0 ± 53.4 mg/dL) groups as compared to the control group (148.5 ± 37.5 mg/dL). A trend of increase in plasma insulin levels was seen with RAPA dosage, but the values did not reach statistical significance. Insulin resistance as defined in humans and calculated by HOMA model was found to be significantly different in the drug-tested groups as compared to the controls (Table 5).

3.3. Hepatic lipids and ACAT activity

There were no differences in hepatic total cholesterol concentrations among the 3 groups (Table 6) tested. Interestingly, the free cholesterol content (unesterfied

Table 7
Total cholesterol, TGs, and TNF-α in aorta of guinea pigs treated with 0 (control), low dose, and high dose of RAPA*

Aorta lipids	Control	Low dose	High dose
TC (mg/g)	11.9 ± 2.0[a]	8.5 ± 2.7[b]	10.8 ± 2.7[a]
TG (mg/g)	16.1 ± 11.0[a]	33.5 ± 12.7[b]	27.6 ± 13.4[b]
TNF-α (pg/mL)	14.5 ± 10.9[a]	52.5 ± 40.3[ab]	151.0 ± 140.9[b]

* Values are mean ± SD for the 10 guinea pigs per group. Values with different superscripts are significantly different as analyzed by 1-way ANOVA and LSD as post hoc test ($P < .01$). Weight gain was used as a covariate.

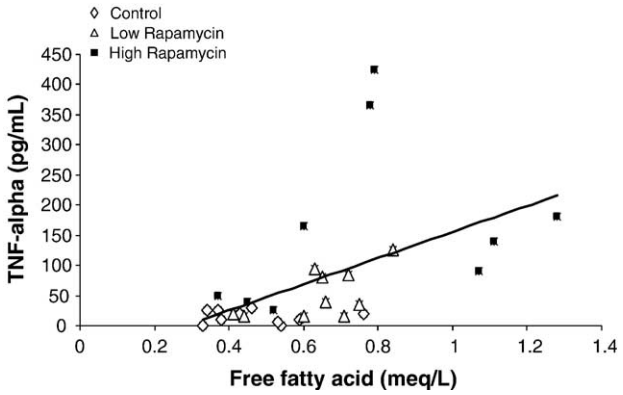

Fig. 3. Correlation between TNF-α and free fatty acid ($r = 0.362$, $P < .05$) of guinea pigs treated with 0 (◇, control), low (Δ), or high (■) doses of rapamycin.

cholesterol) was significantly higher only in the high-dose (1.83 ± 0.56 mg/g) group of RAPA, whereas no difference was observed between the low-dose group (1.39 ± 0.41 mg/g) and the control group (1.43 ± 0.26 mg/g). No significant differences among groups were observed for the esterified cholesterol content in the liver. In addition, TG content in the livers of low RAPA was significantly reduced, whereas no major difference was found in the high RAPA as compared to the controls. Similarly, hepatic ACAT activity was significantly lower in the low-RAPA group ($P < .01$) than in the control and high-RAPA groups (Table 6). Furthermore, a positive correlation was observed between hepatic ACAT activity and the TC content in the aorta (Fig. 2), confirming that hepatic ACAT activity is associated with higher cholesterol content in aortas.

3.4. Aortic lipids and TNF-α

Total cholesterol content in the aorta was significantly lower in the low-dose group (28.6%) than in the control and high-RAPA groups (Table 7). Total cholesterol in the aorta of high-RAPA and control groups was not different. Triglyceride content in the aortas was significantly increased because of RAPA treatment. The low-RAPA group had 51.9% and the high-RAPA group had 41.7% higher TG content in the aorta than the control guinea pigs ($P < .001$).

We also measured TNF-α, a cytokine secreted by macrophages and adipocytes, concentrations in the aortas of these animals. TNF-α values were significantly higher in the 2 tested groups than in the controls (Table 7). Also, an r value of 0.362 between TNF-α and FFA indicates a positive correlation between this inflammatory marker and TG metabolism (Fig. 3).

4. Discussion

Hyperlipidemia has been reported to be present in about 40% to 45% of liver and renal transplant patients receiving RAPA treatment [10,11]. It is not clear whether therapy for hyperlipidemia in transplant patients should follow the guidelines recommended for the general population [31]. It has been suggested that strategies to treat transplant hyperlipidemia should include change in lifestyle such as diet and exercise, reduced doses of immunosuppressive agents and, if necessary, lipid-lowering agents. We selected 2 different doses of RAPA with the intent to study the mechanisms of dyslipidemia associated with RAPA administration. The low dose is equivalent to 10 mg/d, a dose used in renal transplant patients to maintain the therapeutic RAPA (sirolimus) blood at a level of 10 to 15 ng/mL [32] and has been reported to induce hyperlipidemia in renal transplant patients [7,8]. The high dose (0.85 mg/d) is consistent with doses used in animal models [17,18] to allow us to study the mechanisms of RAPA-induced hypertriglyceridemia.

In this study, we demonstrated that RAPA induces hyperlipidemia by interfering/altering triglyceride metabolism to a greater extent than it does cholesterol metabolism in guinea pigs. To our knowledge, this is the first report to document increased TG levels by RAPA administration in the guinea pig model. Plasma TG levels were increased significantly in only 21 days of treatment. In addition, the increase in plasma glucose, FFA, increased deposition of TG in the aorta, and higher concentrations of aortic TNF-α suggest dysregulation of glucose metabolism and the insulin pathway. The guinea pigs receiving the high dose of RAPA (0.85 mg/d for 3 weeks) failed to gain weight, although they consumed the same amount of food. This observation is consistent with the role of mTOR in cell growth (size and mass) and proliferation (cell division) and with the effects of RAPA in reducing cell size and mass by blocking mTOR effects on protein translation [33]. Similarly, studies of S6K1 knockout mice, the direct downstream target of mTOR, which is also inactivated by RAPA, showed decreased cell size, whereas S6K1 overexpression led to increased cell size and mass [33,34].

4.1. Rapamycin and lipoprotein metabolism

Several reports have demonstrated that plasma TG levels increase with RAPA treatment in renal transplant patients [7,35,36]. Similarly, in the current study, we found increases in plasma TG in both groups of guinea pigs taking different doses of RAPA. It has been reported that hypertriglyceridemia induced by RAPA is greater in a shorter period (14 days) as compared to RAPA treatment for a longer period of time (42 days) [7]. In the current study, high plasma TG levels were associated with larger VLDL particles carrying an increased amount of TG when secreted by the liver and, subsequently, a larger LDL particle, which was also shown to have higher TG content as compared to the controls. Furthermore, it was observed that the increase in the mean LDL size was mainly due to decrease in smaller, denser fraction of the LDL. This effect of RAPA can be considered positive because smaller LDL is more susceptible to oxidation and increases the risk of coronary heart disease by 3-fold [37].

The increased plasma TG concentrations in the RAPA treatment groups can be due to increased hepatic production of TG-rich lipoproteins and/or decreased removal of these particles. A study performed in 6 renal transplant patients with sirolimus (RAPA) provided strong evidence that sirolimus intake led to the increased production of TG-rich lipoproteins, which also contributes to the observed hypertriglyceridemia [7]. In this same study, researchers infused $^{13}C_4$-palmitate to prove that there was significant expansion of the FFA pool in the patients taking sirolimus. Similarly, Um et al [38] reported the presence of elevated FFA with high fat feeding in mice lacking S6K-1, the downstream effector of mTOR. Along the same lines, our study also indicates increase in plasma FFA levels. This increase was observed in the high-RAPA group and was accompanied by an increase in glucose and IR.

Hypertriglyceridemia is a major risk factor for cardiovascular disease and is often associated with insulin resistance [39]. An elevated concentration of glucose was also observed by RAPA treatment suggesting that when glucose uptake by cells is not efficient, this leads to more insulin secretion by the pancreas. Interestingly, there was a significant positive correlation between fasting plasma glucose levels and the concentration of TG in VLDL particles, suggesting that lipid and carbohydrate metabolism are being affected simultaneously by RAPA treatment. mTOR is also known to be a key regulator of signals from nutrients, energy, insulin, and growth factors [40]; and RAPA, by inhibiting the activity of mTOR, alters these pathways.

Differences in insulin levels failed to reach statistical significance in the current study mainly because of the use of a mouse antibody, which might not have been specific enough for guinea pigs. Although HOMA formula was originally validated for human studies to estimate insulin sensitivity, use of the HOMA model was recently reported to be a good indicator of IR in animal models [41,42]. Following the same lines and cutoff points as humans, we calculated IR by the HOMA equation [29] for all guinea pigs. Based on this information, the 2 groups of guinea pigs receiving RAPA were found to be insulin resistant. Again, glucose and insulin levels were even higher in the high-RAPA group, indicating that low RAPA is a better dose than high RAPA.

Elevated plasma FFA levels have a major role not only in IR but also in type 2 diabetes [39]. Elevated plasma FFA in obese individuals can be attributed to IR [43]. It is fair to postulate that in the present study the increases in plasma FFA content led to IR as reported by the HOMA equation. Because fasting plasma glucose was also elevated by RAPA treatment, this could be a result of IR.

4.2. Rapamycin and liver and aorta

In an attempt to further understand the mechanism by which RAPA raises plasma TG, we explored the effects of this drug on hepatic and aortic lipids. There were some remarkable differences in hepatic and aortic cholesterol accumulation between the 2 RAPA doses. The low dose of RAPA resulted in less accumulation of free cholesterol in both the liver and the aorta compared to the high RAPA and the control groups. In addition, hepatic ACAT activity was lowest in the low-RAPA group. Because hepatic ACAT activity has been positively correlated with increased atherosclerosis development in the African Green monkey [44], the lower concentrations of ACAT activity and cholesterol in the aorta suggest that the low-RAPA dose is less atherogenic than high-RAPA treatment and provides a better aortic cholesterol profile than the control group. Increased hepatic ACAT activity has been postulated to increase the concentration of CE incorporated into the secreted VLDL, which in turn increases CE in LDL and the potential deposition of CE in the aorta [44]. In guinea pigs, we have also observed that increased ACAT activity is correlated with increases in LDL-C [45]. In the present study, we report a correlation between hepatic ACAT activity and concentrations of CE in aorta which supports these previous findings [44,45].

Aortic TG concentrations, however, were higher in the RAPA groups than in the control. Triglyceride content in aorta has been related to atherosclerosis in some studies [46] and has been demonstrated to be related to hypercholesterolemia. Similarly, in the present study, guinea pigs treated with RAPA had higher TG in plasma as well as in their aorta as compared to the control animals.

Another important parameter that was affected by RAPA treatment was aortic TNF-α concentration, an adipokine secreted by adipocytes and has a cytotoxic effect on tumor cells but not on normal cells. Tumor necrosis factor-α exerts both inflammatory and cell death activity and is also thought to play a role in pathogenesis of atherosclerosis [47]. Although RAPA administration has been shown to decrease TNF-α secretion as a result of inhibition of T-cell proliferation [48], our findings indicate that TNF-α levels are locally increased in the aorta, suggesting that elevated TNF-α concentration is secondary to increased plasma FFA and TG content in the aorta. Similarly, Razeghi et al [49] showed that by using atrophic heart model, TNF-α transcript expression was elevated early and transiently by RAPA treatment. Along the same line, TNF-α has also been detected in human atherosclerotic plaques, and thus it is thought to play a major role in the pathogenesis of coronary artery disease [50]. Furthermore, studies have shown that FFA-induced TNF-α secretion in 3T3-L1 adipocytes incubated with a mixture of fatty acids contributed to cellular IR [51]. Tumor necrosis factor-α is considered a candidate mediator of IR in obesity, is overexpressed in adipose tissue [52], and blocks the action of insulin in cultured cells as well as in humans [53]. Similarly, TNF-α–deficient mice had been shown to have better insulin sensitivity and less FFAs circulating in their plasma [52]. These results are in agreement with the positive correlation found between FFA and TNF-α in the present study.

From these studies, we conclude that TG metabolism was clearly affected by the 2 doses of RAPA tested in guinea

pigs. Rapamycin treatment resulted in elevated concentrations of plasma TG, FFA, glucose, and increased IR. Very low density lipoprotein and LDL particles were significantly modified in size by the incorporation of more TG per particle. Accumulation of TG was also observed in the aorta by RAPA treatment as well as increased production of TNF-α, which was correlated to the elevated FFA.

Many of the measured parameters were similar between low and high doses of RAPA except for weight gain, FFA, TNF-α, and cholesterol accumulation in tissue. Guinea pigs receiving the low-RAPA dose had similar weight gain as the control counterparts. In addition, low doses of RAPA resulted in less aortic cholesterol accumulation compared to high RAPA and nontreated animals, indicating that low doses—if efficacious—present less side effects associated with TG and insulin dysregulation.

Acknowledgment

This work is supported in part by NIH-KO1-DK60654 award to GS.

References

[1] Garber K. Rapamycin's resurrection: a new way to target the cancer cell cycle. J Natl Cancer Inst 2001;93:1517-79.
[2] Brown EJ, Albers MW, Shin TB, Ichikawa K, et al. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature 1994;369:756-8.
[3] Hay N, Sonnenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18:1926-45.
[4] Jaeschkle A, Dennis PB, Thomas G. mTor a mediator of intracellular homeostasis. Curr Top Microbiol Immunol 2004;279:283-98.
[5] Tee AR, Blenis J, Proud CG. Analysis of mTOR signaling by the small G proteins Rheb and Rheb-1. FEBS Lett 2005;579:4763-8.
[6] Mathis AS, Dave N, Knipp GT, Friedman GS. Drug-related dyslipidemia after renal transplantation. Am J Health Syst Pharm 2004;61:565-85.
[7] Morrisett JD, Abdel-Fattah G, Hoogeven R, Mitchell E, et al. Effects of sirolimus on plasma lipids, lipoprotein levels, and fatty acid metabolism in renal transplant patients. J Lipid Res 2002;43:1170-80.
[8] Hoogeven RC, Ballantyne CM, Pownall HJ, Opekun AR, et al. Effect of sirolimus on the metabolism of apoB100-containing lipoproteins in renal transplant patients. Transplantation 2001;72:1244-50.
[9] MacDonald AS. Rapamycin in combination with cyclosporine or tacrolimus in liver, pancreas and kidney transplantation. Transplant Proc 2003;35:201S-8S.
[10] Mathe D, Adam R, Malmendier C, Gigou M, et al. Prevalence of dyslipidemia in liver transplant recipients. Transplantation 1992;54:16.
[11] Kahan BD, Yakupoghlu YK, Schoenberg L, et al. Low incidence of malignancy among sirolimus/cyclosporine-treated renal transplant recipients. Transplantation 2005;80:749-58.
[12] Galanis E, Buckner JC, Mauer MJ, et al. Phase II trial of temsirolimus (CCI-779) in recurrent glioblastoma multiforme: a North Central Cancer Treatment Group Study. J Clin Oncol 2005;23:5294-304.
[13] Fernandez ML. Guinea pigs as models for cholesterol and lipoprotein metabolism. J Nutr 2001;131:10-20.
[14] Conde K, Pineda G, Newton R, Fernandez ML. Hypocholesterolemic effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors in the guinea pig. Atorvastatin versus simvastatin. Biochem Pharmacol 1999;58:1209-19.
[15] West KL, Zern TL, Butteinger D, Keller BT, Fernandez ML. SC-435, an ileal apical sodium co-dependent bile acid transporter (ASBT) inhibitor lowers plasma cholesterol and reduces atherosclerosis in guinea pigs. Atherosclerosis 2003;171:201-10.
[16] Aggarwal D, West KL, Zern TL, Shresthra S, Vergara-Jimenez M, Fernandez ML. JTT-130, a microsomal transfer protein inhibitor lowers plasma triglycerides and LDL cholesterol concentrations without increasing hepatic triglycerides in the guinea pig. BMC Cardiovasc Disord 2005;5:30.
[17] Dejneka NS, Kuroki AM, Fosnot J, Tang W, Tolentino MJ, Bennett J. Systemic rapamycin inhibits retinal and choroidal neovascularization in mice. Mol Vis 2004;10:964-72.
[18] Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med 2002;8:128-35.
[19] Lin ECK, Fernandez ML, McNamara DJ. Dietary fat type and cholesterol quantity interact to affect cholesterol metabolism in guinea pigs. J Nutr 1992;122:2019-29.
[20] Fernandez ML, Wilson TA, Conde K, Vergara-Jimenez M, Nicolosi RJ. Hamsters and guinea pigs differ in their plasma lipoprotein cholesterol distribution when fed diets varying in animal protein, soluble fiber or cholesterol content. J Nutr 1999;129:1323-32.
[21] Allain CC, Poon LS, Chan CS, Richard W, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem 1974;20:470-5.
[22] Carr T, Anderssen CJ, Rudel LL. Enzymatic determination of triglycerides, free cholesterol, and total cholesterol in tissue lipid extracts. Clin Biochem 1993;26:39-42.
[23] Zern T, West KL, Fernandez ML. Grape polyphenols decrease plasma triglycerides and reduce cholesterol accumulation in the aorta of ovariectomized guinea pigs. J Nutr 2003;133:2268-72.
[24] Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 1978;87:206-10.
[25] Homquist L, Carlson K, Carlson LA. Comparison between the use of isopropanol and tetramethylurea for the solubilization and quantitation of human serum very low density apolipoproteins. Anal Biochem J 1987;149:423-6.
[26] Van Heek M, Zilversmit D. Mechanisms of hypertriglyceridemia in the coconut oil/cholesterol-fed rabbits increased secretion and decreased catabolism of VLDL. Arteriosclerosis Thromb 1991;11:918-27.
[27] Smith JL, de Jersey J, Pilay SP, Hardy IR. Hepatic acyl-CoA: cholesterol acyltransferase. Development of a standard assay and determination in patients with cholesterol gallstones. Clin Chim Acta 1986;158:271-82.
[28] Reljic R, Ries M, Anic N, Ries B. New chromogen for assay of glucose in serum. Clin Chem 1992;38:522-5.
[29] Haffner SM, Miettinen H, Stern P. The homeostasis model in the San Antonio Heart Study. Diabetes Care 1997;120:1087-92.
[30] Alexaki A, Wilson T, Attalah MT, Handelman G, Nicolosi RJ. Diets high in saturated fat have increased cholesterol accumulation and cytokine production in the aortic arch compared with cholesterol-fed hamsters with moderately elevated plasma non-HDL cholesterol concentrations. J Nutr 2004;134:410-5.
[31] Kniepeiss D, Iberer F, Schafellner S, Jakoby E, et al. Dyslipidemia during sirolimus therapy in patients after liver transplantation. Clin Transplant 2004;18:642-6.
[32] Benavides C, Mahmoud KH, Knight R, et al. Rabbit antithymocyte globulin: a postoperative risk factor for sirolimus-treated renal transplant patients? Transplant Proc 2005;37:822-6.
[33] Pearson RB, Dennis PB, Jan JW, Williamson NA, et al. The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site with a conserved hydrophobic domain. EMBO J 1995;14:5279-87.
[34] Fingar DC, Richardos CJ, Tee AR, Cheatham L, et al. mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factors 4E. Mol Cell Biol 2004;24:200.

[35] Brattstrom C, Wilczek HE, Tyden G, Bottiger Y, et al. Hyperlipidemia in renal transplant recipients treated with sirolimus (rapamycin). Transplantation 1998;65:1273-4.
[36] Brattstrom C, Wilczek HE, Tydern G, Bottiger Y, et al. Hypertriglyceridemia in renal transplant recipients treated with sirolimus. Transplant Proc 1998;30:3950-1.
[37] Krauss RM. Atherogenic lipoprotein phenotype and diet-gene interactions. J Nutr 2001;131:340S-3S.
[38] Um HS, Frigerio F, et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature 2004; 431:200-5.
[39] Ginsberg H. Insulin resistance and cardiovascular disease. J Clin Invest 2000;106:453-8.
[40] Soliman G. The mammalian target of rapamycin signaling network and gene regulation. Curr Opin Lipidol 2005;16:317-23.
[41] Appleton DJ, Rand JS, Sunvold GD. Basal plasma insulin and homeostasis model assessment (HOMA) are indicators of insulin sensitivity in cats. J Feline Med Surg 2005;7:183-93.
[42] Pickavance LJC, Brand CL, Wasserman K, Wilding JP. The dual PPAR alpha gamma/agonist ragaglitazar improves insulin sensitivity and metabolic profile equally with pioglitazone in diabetic and dietary obese ZDF rats. Br J Pharmacol 2005;144:308-16.
[43] Boden G. Effects of free fatty acids on glucose metabolism: significance for insulin resistance and type 2 diabetes. Exp Clin Endocrinol Diabetes 2003;111:121-4.
[44] Carr TP, Parks JS, Rudel LL. Hepatic ACAT activity in African green monkey is highly correlated to plasma LDL cholesteryl ester enrichment and coronary artery atherosclerosis. Arterioscler Thromb 1992;12:1274-83.
[45] Fernandez ML, McNamara DJ. Dietary fat saturation and chain length modulate guinea pig hepatic cholesterol metabolism. J Nutr 1994;124:331-339.
[46] Ahmad-Raus RR, Abdul-Latif ES, Mohammad JJ. Lowering of lipid composition in aorta of guinea pigs by *Curcuma domestica*. BMC Complement Altern Med 2001;1:6.
[47] Boesten LS, Zadelaar AS, van Nieuwkoop A, Gijbels MK, et al. Tumor necrosis factor-[alpha] promotes atherosclerotic lesion progression in APOE*3-leiden transgenic mice. Cardiovasc Res 2005;66:179-85.
[48] Adkins JR, Castresana MR, et al. Rapamycin inhibits release of tumor necrosis factor–alpha from human vascular smooth muscle cells. Am Surg 2004;70:384-7.
[49] Razeghi P, Bruckner BA, Sharma S, Yonker FA, et al. Mechanical unloading of the failing human heart fails to activate the protein kinase B/Akt/glycogen synthase kinase-3beta survival pathway. Cardiology 2003;100:17-22.
[50] Barath P, Fishbein M, Vari S, Forrester JS. Detection and localization of tumor necrosis factor in human atheroma. Am J Cardiol 1990;65: 297-302.
[51] Nguyen MTA, Atoh H, Favelyukis S, Babendure JL, et al. JNK and TNF-α mediate free fatty acid–induced insulin resistance in 3T3-L1 adipocytes. J Biol Chem 2005;280:35361-71.
[52] Kern PA, Saghizadeh M, Ong JM, Bosch RJ, et al. The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. J Clin Invest 1995; 95:2111-9.
[53] Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature 1997;389:610-4.