SEARCH FOR EXTRAPANCREATIC EFFECTS OF NEW ORAL HYPOGLYCEMIC AGENT A-4166: 1. ORAL GLUCOSE TOLERANCE TESTS IN NORMAL AND HEREDITARY INSULIN RESISTANT RATS

I. KLIMES, E. SEBOKOVA, D. GASPERIKOVA, A. MITKOVA, S. KUKLOVA, P. BOHOV, J. STANEK[1]

Diabetes and Nutrition Research Group, Institute of Experimental Endocrinology, Slovak Academy of Sciences, 833 06 Bratislava, Slovakia; [1]Clinic of Gastroenterology, Institute of Postgraduate Medical Education, Bratislava

Objective. To test the effect of new oral hypoglycemic compound A-4166 on insulin secretion during oral glucose challenge in normal and hereditary non-obese, hypertriglyceridemic, insulin resistant and hypertensive rats fed either a normal or high fat diet.

Methods. The rats used were 15 weeks old males of Wistar Charles River strain (controls) and Wistar-derived hereditary hypertriglyceridemic (hHTg) rats of our own colony. They were fed either basal (12 cal% of fat) or high fat diet (70 cal% fat). After 3 weeks of feeding the above diets, the oral glucose tolerance tests (2 g/kg) were carried out in unrestrained conscious rats kept in special metabolic cages after overnight fasting and ten minutes after the administration of A-4166 (100 mg/kg) or placebo by the stomach tube. Plasma glucose, triglycerides, free fatty acids and insulin levels were measured by routine analytical methods.

Results. High fat diet feeding resulted in an increase in fasting plasma insulin in both rat strains, while fasting plasma glucose in high fat diet fed animals remained unchanged as compared to those fed basal diet. No differences in the fasting FFA levels were found. The glucose area under curve (AUC) did not differ between the two strains used and high fat diet resulted in a higher glucose AUC in both strains. The administration of A-4166 improved the glucose tolerance in all animals, namely in those fed the basal diet. Insulin AUC showed very similar pattern in both rat strains proving the stimulatory effect of A-4166 on insulin secretion during an oral glucose challenge. High fat feeding resulted in an impairment of insulin action, but the administration of A-4166 restored the antilipolysis in both strains to the normal range.

Conclusions. The previously reported hypoglycemic action of A-4166 resulting from the increased insulin secretion was confirmed. Moreover, some beneficial action of A-4166 on antilipolysis *in vivo* was demonstrated.

Key words: Insulin - OGTT - Glucose - FFA - Hereditary insulin resistant rats - Hypoglycemic drug A-4166 -High fat diet

Non-insulin-dependent diabetes mellitus (NIDDM) is a metabolic disease which affects almost 10 % of the aging society in modern Western countries (VALLE et al. 1997). NIDDM is a heterogenic disorder, and may appear as almost different diseases in different patients. Generally the defects in NIDDM are characterized by a reduced action of insulin (insulin resistance) and insufficient insulin secretion (relative insulin deficiency), resulting in hyperglycemia (DEFRONZO et al. 1997).

Patients with persisting hyperglycemia have increased risk of developing microvascular and macrovascular complications (LEROITH et al. 1996). Thus, a tight glucose control is the ultimate goal also in treatment of NIDDM patients. An effective therapy must target either the insulin secretory capacity of the beta cells, the insulin action in peripheral tissues or both.

The normal physiologic insulin response to a nutritional stimulus is rapid and transient. This serves

for inhibition of the hepatic glucose production, promotion of the hepatic glucose uptake and stimulation of utilization of the nutrient load by peripheral tissues. Therefore, a drug capable of mimicking or restoring early insulin release would be a most attractive therapy for NIDDM.

The D-phenylalanine derivative, A-4166, is a compound which has been found to stimulate insulin secretion in mice, rats and dogs with a rapid onset and short duration of action (Shinkai and Sato 1990; Sato et al. 1991). With oral administration, the A-4166 is rapidly absorbed and greatly improves glucose tolerance, without causing hypoglycemia. Moreover, when administered by gavage 10 minutes prior to oral glucose challenge, the A-4166 reduced glucose elevations in normal rats fed a high fat diet to induce insulin resistance (Anonymous 1995).

The relationship between dietary fat intake and insulin action is nowadays well accepted (Storlien et al. 1996). High fat feeding leeds to insulin resistance in the liver and in peripheral tissues including white and brown adipose tissues, and, more importantly, in a range of skeletal muscles. Beside the amount also the type of dietary fat is critical for the impairment of insulin action. The mechanism of changes after high fat feeding are still not fully unravelled. Among the factors that have shown to possibly play a role, is the accumulation of triglycerides in muscle tissues (Fryer and Kruszynska 1993). Stored triglycerides may inhibit glucose metabolism in skeletal muscle through the operation of the Randle's glucose-fatty acid cycle.

The effects of high fat feeding on insulin secretion have received little attention. In the face of the induced insulin resistance ensuing the ingestion of high fat diet, insulin secretion can be expected to be increased, to compensate for the reduced insulin action (Wiersma 1997). In spite of this, a limited number of studies that have looked at glucose-induced insulin secretion after high fat diet feeding have suggested the contrary (Portha et al. 1982; Takahashi et al. 1991; Mlekusch et al. 1991).

Thus, we felt tempted to test the stimulatory action of the novel hypoglycemic compound, A-4166, on insulin secretion during an oral glucose challenge in normal rats fed either a standard lab chow or a high fat diet (known to induce insulin resistance) using also placebo-treated control groups for both diets. Moreover, the identical protocol was applied to a non-obese animal model of endogenous hypertriglyceridemia, insulin resistance and hypertension, i.e. to the hHTg rat (Vrana and Kazdova 1990; Klimes et al. 1994; Klimes et al. 1995). This model of the insulin resistance syndrome was used in order to prove the effectiveness of A-4166 in an insulin resistance state.

Materials and Methods

Animals and diets: Male Wistar Charles River rats (AnLab, Prague, Czech Republic) aged 15 weeks were used as control animals and Wistar-derived hHTg (hereditary hypertriglyceridemic rats of our own colony) were used as the insulin resistant animals. Always four animals were housed per one wire mesh cage in a temperature (22±2 °C) and light controlled room (12 h light:dark cycle; lights on at 6.00 h), and randomly assigned in groups by 12 animals each to feeding two different diets *ad libitum* for 3 weeks: 1. commercial (VELAZ, Prague, Czech Republic) standard laboratory rat chow ST 1 (onward called "basal" diet) containing 12 cal% of fat; 2. high fat diet consisting of 70 cal% fat and prepared according Storlien et al. (1991). For the detailed fatty acid composition of both diets see Tab. 1. The lipids were extracted from the diets (Bligh and Dyer 1959) and methyl esters were prepared after alkaline hydrolysis of lipids by esterification with diazomethane. The fatty acid determination was performed by gas-liquid chromatography (Finnigan, model 9001, Austin, Texas) with flame ionization detection using an SP 2340 fused silica capillary column (Supelco, Bellefonte, PA, USA). Data were quantified based on heneicosanoid acid (21:0) as an internal standard with the aid of the CSW 1.6 chromatography station software (Data Apex, Prague, Czech Republic).

Oral glucose tolerance tests and the mode of A-4166 administration: After three weeks of feeding the above described diets, rats were anesthetized by i.p. injection of xylazine hydrochloride (10 mg/kg) plus ketamine hydrochloride (75 mg/kg) and fitted with chronic artery cannula. The oral glucose tolerance tests (2 g/kg) were carried out after 16 hours of overnight food deprivation in unrestrained conscious

Table 1
Fatty acid composition of experimental diets (wt%)

FATTY ACID	BASAL DIET	HIGH FAT DIET
14:0	0.9 ± 0.05	1.6 ± 0.01
16:0	21.9 ± 0.10	21.4 ± 0.10
16:1 n-7	1.8 ± 0.05	1.4 ± 0.01
18:0	7.6 ± 0.01	15.7 ± 0.10
18:1 n-9	25.3 ± 0.04	32.5 ± 0.10
18:1 n-7	1.8 ± 0.01	2.0 ± 0.01
18:2 n-6	32.0 ± 0.03	20.0 ± 0.30
18:3 n-3	4.2 ± 0.03	0.5 ± 0.01
20:4 n-6	0.2 ± 0.01	0.1 ± 0.01
20:5 n-3	0.04 ± 0.0	N.D.
22:5 n-3	0.06 ± 0.0	0.03 ± 0.0
22:6 n-3	0.05 ± 0.0	N.D.
Others	4.2	3.9
Total		
SFA	32.2 ± 0.07	40.9 ± 0.02
MUFA	30.3 ± 0.06	37.1 ± 0.10
PUFA n-6	32.5 ± 0.02	20.2 ± 0.03
PUFA n-3	4.3 ± 0.04	0.6 ± 0.01
n-6/n-3	7.5 ± 0.06	36.9 ± 0.80

SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, N.D. = not detectable

rats kept in special metabolic cages. Ten minutes prior to oral glucose challenge the active substance A-4166 (100 mg/kg or the same amount of placebo) was administered by the stomach tube. As the A-4166 is not water soluble, a suspension of A-4166 in 0.5 % methylcellulose in water was achieved with the aid of a Polytron homogenizer. In the placebo experiments, a corresponding volume (to body weight adjusted) of 0.5 % methycellulose without the active substance added was used. Yet prior to drug (or placebo) administration (-10 minutes) and then at 0, 15, 30, 60 and 120 minutes after oral glucose load the blood was withdrawn from the arterial cannula and the plasma obtained after centrifugation was used for the analyses of glucose, insulin and free fatty acid levels.

Routine analytical methods: Plasma glucose (GLU, Boehringer Mannheim, Germany), triglycerides (TG MPR2, Boehringer Mannheim, Germany) and serum free fatty acid concentrations (FFA, Randox Ardmore, UK) were measured with the aid of specific commercially available kits using the BM Hitachi 704 (Japan) biochemical autoanalyzer. Plasma insulin levels were measured using the Rat Insulin Ria Kit (Linco Research Inc., St. Charles, MO, USA).

Statistical evaluation: Differences between groups were evaluated using the analysis of variance (SPSS/PC + software). The univariate ANOVA was used for parameters obtained before any drug or placebo had been administered. All other data were analyzed by multivariate ANOVA. Threshold for significance was set to $P<0.05$.

Results

Animal characteristics: As expected, the initial body weight of control animals was significantly higher (393±15 g) than that of hHTg rats of the same age (264±8 g, $P<0.001$). Even the final weight after three weeks of feeding was higher in controls (437±12 vs. 286±5 g). Although the average body weight gain was higher in controls (44 g) than in hHTg animals (22 g), the difference was not significant. There were no significant differences in body weight increments of both rat strains fed either basal or high fat diet diets except that the body weight increments were consistently lower in the hHTg rats, and the hHTg rats gained more weight on the high fat diet than at the basal chow (Tab. 2).

Fasting plasma glucose levels were not influenced by feeding the high fat diet in both strains under study (Tab. 2). Nevertheless, the hHTg rats had higher fasting plasma glucose levels than the controls, although these values remained within normal limits. The hHTg rats had lower fasting insulinemia when compared to control rats, and feeding the high fat diet resulted in an increase in fasting plasma insulin in both rat strains studied (Tab. 2). As fasting plasma glucose remained unchanged in high fat fed animals, the higher fasting insulinemia may reflect a compensatory response of the B cells to a high fat diet-induced impairment of insulin action in the hHTg rats. Feeding the high fat diet to both rat strains was accompanied with no substantial changes in the fasting serum FFA levels (Tab. 2).

Glucose tolerance: The results of oral glucose tolerance tests (oGTT) which were carried out 10 min after the administration of A-4166 (100 mg/kg in 0.5 % methylcellulose) or an equivalent volume of methylcellulose alone (as placebo) by oral gavage are shown in Tab. 3. The glucose area under curve (AUC) did not differ between the two rat strains used, and high fat diet resulted in a similar worsening of

Table 2
Animal characteristics*

	CONTROL				hHTg			
	BD		HF		BD		HF	
	A-4166	Placebo	A-4166	Placebo	A-4166	Placebo	A-4166	Placebo
BW[1] incr.	44±6.9	45±5.8	47±3.5	55±5.8	16±2.0	17±2.0	25±2.7	26±2.3
FPG[2]	5.8±0.2	5.2±0.3	5.8±0.3	5.3±0.2	6.0±0.2	5.9±0.3	6.4±0.2	5.8±0.3
FPI[3]	59±18	65±15	75±15	78±11	42±5	33±4	50±6	54±8
FFFA[4]	0.9±0.07	1.0±0.09	0.8±0.05	0.7±0.03	0.7±0.07	0.9±0.04	0.9±0.04	1.0±0.05

*All parameters reported above relate to the status 3 weeks after feeding either the basal or the high fat diets yet BEFORE any drug or placebo was administered

[1]BW incr.= body weight increment [g], [2]FPG = fasting plasma glucose [mmol.l^{-1}], [3]FPI = fasting plasma insulin [μU.ml^{-1}], [4]FFFA = fasting serum free fatty acids [mmol.l^{-1})

Test of significance for	BW incr	FPG	FPI	FFFA
Source of variation:		Significance of F		
animal	.000	.046	.006	
diet	.010		.090	

Table 3
Oral glucose tolerance test after 3 weeks of dietary treatment in control and insulin resistant hHTg rats: Area-under-curve /AUC/ values in percentage for plasma glucose, insulin, FFA and the OR

	CONTROL				hHTg			
	BD		HF		BD		HF	
	A-4166	Placebo	A-4166	Placebo	A-4166	Placebo	A-4166	Placebo
Glucose AUC (%) x 10^{-2}	135±7	183±7	171±6	198±8	135±7	170±5	173±14	193±6
ins AUCx10^{3} (%) x 10^{-2}	547±80	339±23	292±37	249±35	336±30	286±37	350±41	297±51
OR[1] 3.8±0.8	1.9±0.2	1.6±0.2	1.4±0.3	2.8±0.3	1.6±0.3	2.3±0.6	1.7±0.3	
FFA[2] AUC (%)x10^{-2}	82±6	79±6	94±5	100±4	123±23	105±7.5	105±7.5	113±11

[1]OR = The overall ratio /over 120 min) is calculated by dividing the insulin AUC by the glucose AUC. [2]FFA = Free fatty acids

Test of significance for:	AUC:	Glucose	Insulin	FFA and	OR
Source of variation:			Significance of F		
animal				.002	
diet		.000			.005
drug		.000	.000		.001

the glucose tolerance as shown by higher glucose AUC of control and hHTg rats (Tab. 3 and Fig. 1). The oral administration of A-4166 prior to glucose challenge improved significantly the glucose tolerance in all animals studied with a more pronounced action in rats fed the basal diets (Tab. 3, Fig. 1).

Closer evaluation of the glycemia time courses during the oGTTs showed that the peak levels of glycemia in both the control and hHTg rats treated with A-4166 were much lower than these in the respective placebo treated groups. Moreover, the glycemic curves were much flatter with A-4166 indicating that

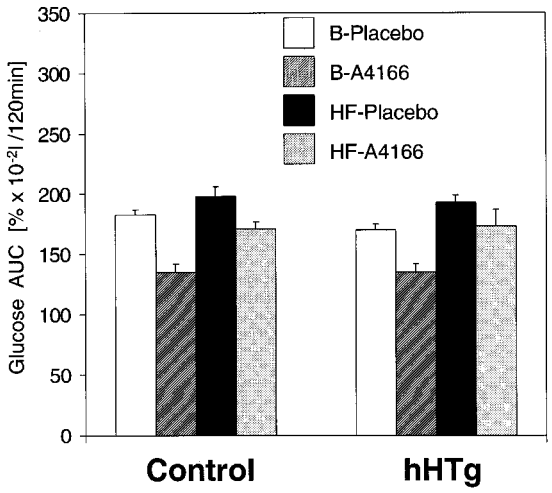

Fig. 1
Oral glucose tolerance test after 3 weeks of dietary treatment in control and insulin resistant hHTg rats: Area-under-curve (AUC) values in percentage for plasma glucose. Values are means±SEM. For statistical significance see the legend to Tab. 3.
B - basal diet, HF - high fat diet

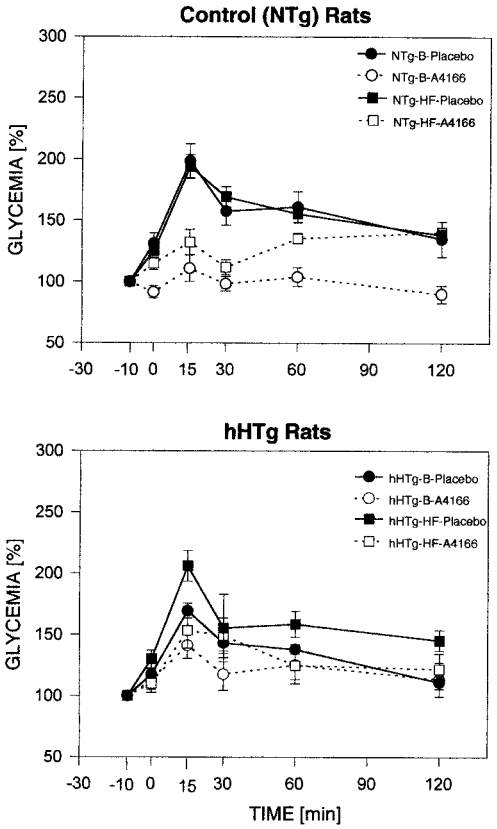

Fig. 2
Plasma glucose concentrations during oGTT in control (upper panel) and insulin resistant hHTg (lower panel). Values are means±SEM. Test of significance for peak plasma glucose level (+15 min):
B - basal diet, HF - high fat diet

Source of variation:	Significance of F:
animal	
diet	
drug	0.000

this drug eliminates the sharp post-challenge glycemic excursions (Fig. 2).

Insulin AUC showed very similar pattern in both rat strains proving the stimulatory effect of A-4166 on insulin secretion during an oral glucose challenge. It should be added that the most pronounced A-4166 action on insulin secretion was found in control rats fed the basal diet (Tab. 3 and Fig. 3). The obtained time courses of insulinemia during oGTTs confirmed a rapid onset and short duration of the stimulatory action of A-4166 on insulin secretion in both control and the insulin resistant hHTg rats (Fig. 4).

Dividing the insulin AUC by the glucose AUC (overall ratio /OR/,120 min) corrects for differences in obtained blood glucose levels during oGTTs. The OR confirmed lower insulin secretion in control and hHTg animals fed the high fat diet. Moreover, it reconfirmed the stimulatory action of A-4166 on insulin secretion as shown in Tab. 3, Fig. 3 and 4.

Free fatty acid AUC (Tab. 3 and Fig. 5): In the control rats no differences were observed whether in the response to feeding the high fat diet or due to A-4166 treatment. The hHTg rats had significantly higher FFA AUC than the control group but the overall pattern did not change in response either to diet or drug treatment The individual time courses of serum FFA suppression are shown in Fig. 6. They demonstrate that the slope of the curve for serum FFA levels in the early phase of the oGTT is shifted to the right in both the control and hHTg rats fed the high fat diet which received placebo. This shows an impairment of insulin action by high fat feeding. The aforementioned is further supported by lower serum FFA decrements in the early phase of the oGTT (Tab. 4). The single dosage of A-4166 administered 10 min before the glucose challenge restored the antilipolysis in both strains back to the normal range (Tab. 4).

Table 4
Oral glucose tolerance test after 3 weeks of dietary treatment in control and insulin resistant hHTg rats: Decrements of serum FFA * and increments of plasma insulin * * in percentage during early phase of the oGTT

	CONTROL				hHTg			
	BD		HF		BD		HF	
	A-4166	Placebo	A-4166	Placebo	A-4166	Placebo	A-4166	Placebo
ΔIns (%)	714±165	404±97	434±108	267±71	390±80	333±59	317±51	367±82
ΔFFA (%)	46±7	44±7	48±4	19±9	40±15	35±8	34±7	18±9

*Decrements in serum FFA levels were calculated as the difference of serum FFA at -10 min before the commencement of the oGTT and FFA levels at +15 min after administration of the glucose load.
**Increments in plasma insulin were calculated as the difference of plasma Ins at -10 min before the commencement of the oGTT and Ins levels at +15 min after administration of the glucose load.

Test of significance for:	ΔIns	ΔFFA
Source of variation:	Significance of F	
animal		
diet		.064
drug	.090	.039

Discussion

A high percentage of patients with NIDDM are currently treated with oral hypoglycemic agents which affect different steps of the glucose metabolism. There are several classes of the oral antidiabetic drugs. The sulfonylurea (SU) derivatives realize their hypoglycemic action by stimulating the insulin secretion from the B cells of the islets of Langerhans. Nevertheless, it has been shown that SU derivatives have also some extrapancreatic effects increasing the insulin action in its target tissues (Bak et al. 1989). The biguanide derivatives - e.g. metformin - reduce glycemia without stimulating the insulin secretion where the glucose lowering effects result mainly from increased glucose utilization (Bailey 1992). A new class of antidiabetic agents - the thiazolidinediones appear to increase insulin sensitivity and responsiveness in target tissues, improve glucose tolerance with not much effect on insulin levels (Bressler and Johnson 1992).

Several new non-sulfonylurea hypoglycemic agents (e.g. repaglinide, KAD-1229 or A-4166), structurally related to meglitinide, show a potent stimulatory effect on insulin secretion from B cells and markedly augment glucose stimulated insulin release (Malaisse 1995). Nevertheless, their possible extrapancreatic effects have not been fully clarified as yet. To test that we have set up a protocol exploiting the euglycemic hyperinsulinemic clamp technique with double labelled glucose administration, and a bolus dose of A-4166 or placebo to normal and endogenously insulin resistant hHTg rats. Nevertheless, before launching

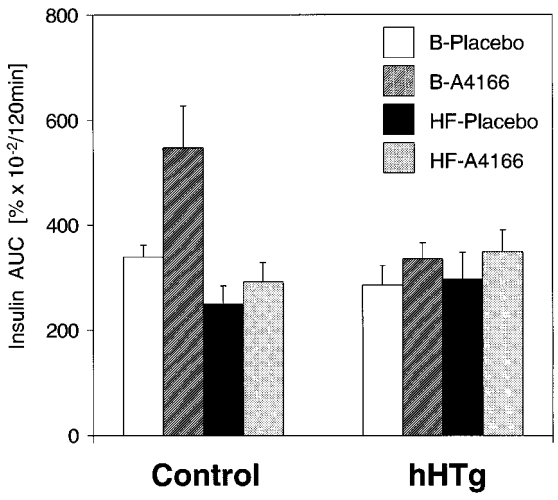

Fig. 3
Oral glucose tolerance test after 3 weeks of dietary treatment in control and insulin resistant hHTg rats: Area-under-curve (AUC) values in percentage for plasma insulin. Values are means±SEM. For statistical significance see the legend to Tab. 3.
B - basal diet, HF - high fat diet

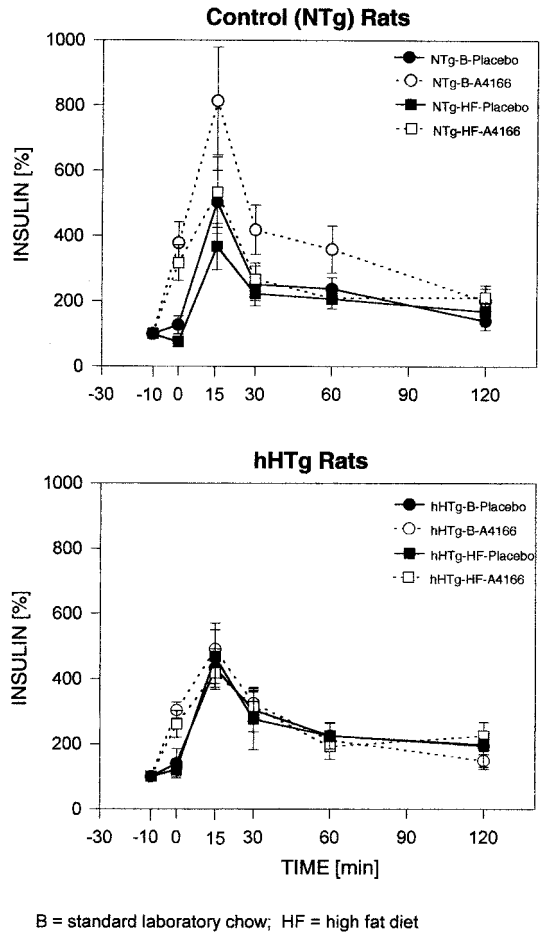

Fig. 4
Plasma insulin concentrations during oGTT in control (upper panel) and insulin resistant hHTg (lower panel) rats. Values are means±SEM.
B - basal diet, HF - high fat diet

Test of significance for peak plasma insulin level (+15 min):

Source of variation:	Significance of F:
animal	
diet	
drug	0.000

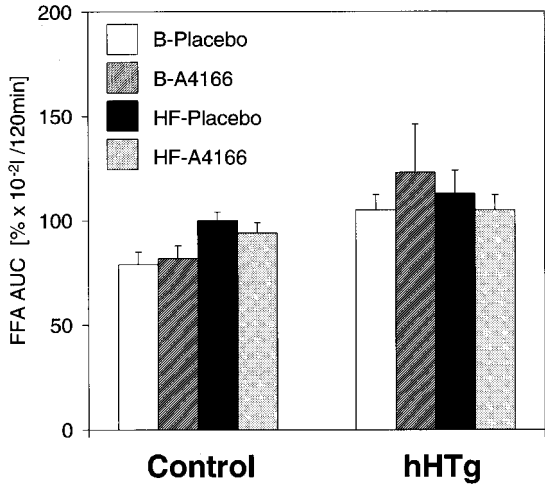

Fig. 5
Oral glucose tolerance test after 3 weeks of dietary treatment in control and insulin resistant hHTg rats: Area-under-curve (AUC) values in percentage for serum free fatty acids. Values are means±SEM. For statistical signficance see the legend to Tab. 3.
B - basal diet, HF - high fat diet

the aforementioned large study, we felt tempted to verify the A-4166 hypoglycemic action in normal and insulin resistant rats.

Our recent data confirm the earlier observations on a rapid and short lasting stimulatory action of A-4166 on insulin secretion (Shinkai and Sato 1990; Sato et al. 1991). These changes were observed not only in Wistar control rats but also in the insulin resistant hHTg animals. The increases in the amounts of secreted insulin during the 120 minutes of the oGTT went mostly at the account of an augmented early phase of insulin release.

As far as the improvement of glucose tolerance after a single dose of A-4166 is concerned, we were able to reproduce this kind of data in all animals studied regardless to the dietary treatment. Moreover, closer evaluation of the glycemia time courses during the oGTTs showed that the glycemic curves were much flatter with A-4166. This indicates that this molecule removes the sharp post-challenge glycemic excursions. This feature of the A-4166 hypoglycemic action is very valuable in view of the deleterious effects of hyperglycemia on chronic diabetic complications (Yki-Jarvinen 1992; DeFronzo et al. 1997).

Another favorable observation aroses from analyses of the serum FFA levels during the oGTTs. It was shown that the high fat diet-induced reduction in insulin-induced antilipolysis (Sebokova and Klimes 1997) (being evaluated as decrements in serum FFA during the early phase of the oGTT) which was present in both control and hHTg rats, can be also overcome by A-4166 gavage preceeding the glucose load. Our recent data does not,

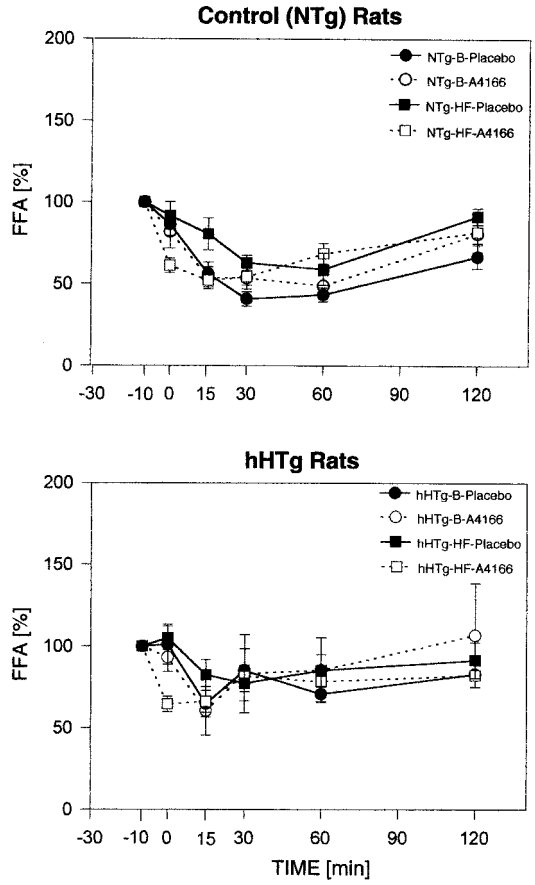

Fig. 6
Serum FFA concentrations during oGTT in control (upper panel) and insulin resistant hHTg (lower panel) rats. Values are means±SEM.
B - basal diet, HF - high fat diet

Test of significance for peak suppression of FFA level (+15 min):

Source of variation:	Significance of F:
animal	
diet	0.019
drug	0.000

however, allow us to answer the question whether this phenomenon is due solely to A-4166 induced increase in insulin secretion or due to some extrapancreatic effects of this molecule on lipolysis per se. Based on a lack of correlation (data not shown) between the FFA decrements and insulin increments in the early phase of the oGTTs (Tab. 4), it seems that the A-4166 induced potentiation of the glucose mediated insulin release should not be made responsible for the aforementioned phenomenon.

Taken together, we have confirmed the earlier reported hypoglycemic action of A-4166 which results from the raised insulin secretion. Moreover, we have shown some beneficial action of A-4166 on antilipolysis *in vivo.*

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Corresponding Author: Iwar Klimes, M.D., DrSc.
Diabetes and Nutrition Research Group
Institute of Experimental Endocrinology
Slovak Academy of Sciences
Vlarska 3
833 06 Bratislava, Slovakia
Phone: +421-7-372687
Fax: +421-7-372687
E-mail: ueeniwar@savba.savba.sk

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