EFFECTS OF NEW HYPOGLYCEMIC AGENT A-4166 ON LIPOLYSIS AND LIPOGENESIS IN RAT ADIPOCYTES

MACHO L., FICKOVA M., ZORAD S., SEBOKOVA E., KLIMES I.

Institute of Experimental Endocrinology, Slovak Academy of Sciences, 833 06 Bratislava, Slovakia
E-mail: ueenmach@savba.savba.sk

Objective. To test the effects of novel oral hypoglycemic agent A-4166 on lipolysis and lipogenesis in adipocytes from normal rats and non-obese, hypertriglyceridemic, insulin resistant and hypertensive rats (HTG) fed basal or high fat diet.

Methods. Adult male Wistar rats and hereditary HTG rats (from our own colony) were used. They were fed either basal or high fat diet for three weeks. On the day of observation the active substance A-4166 was administered intragastrically by gavage 30 minutes before decapitation. Blood was collected for the determination of insulin, glycemia, non esterified fatty acids (NEFA) by using commercial kits. The isolated adipocytes were prepared from epididymal fat pads and lipolysis (by measurement of glycerol release) and lipogenesis (by estimation of labeled glucose incorporation into lipids) were determined.

Results. The administration of A-4166 results in increased serum insulin and decreased serum glucose level in all rats irrespective of the diet. A significant diminution of serum NEFA levels was observed in A-4166 administered Wistar and HTG rats fed high fat diet. In both groups of rats fed basal diet the lipolysis was not affected by A-4166. However, a decrease of lipolysis was found after A-4166 in Wistar rats fed high fat diet. The stimulation of lipolysis by norepinephrine was not influenced by A-4166. A lowered basal lipolysis was found in HTG rats fed high fat diet. The stimulation of lipolysis by norepinephrine was diminished in HTG rats as compared to Wistar animals. Administration of A-4166 did not affect the stimulation of lipolysis by norepinephrine in HTG rats. A decrease of stimulatory action of insulin on lipogenesis was found in Wistar rats fed high fat diet and in all groups of HTG rats. The administration of A-4166 did not change the basal lipogenesis and also the effect of insulin on lipogenesis .

Conclusions. Besides the hyperinsulinemic and hypoglycemic effect of A-4166 also an influence on nonesterified fatty acid serum levels was observed in rats fed high fat diet. This can be partially explained by an antilipolytic action of hyperinsulinemia after A-4166. The studies of lipogenesis showed that Wistar rats fed high fat diet and HTG animals are resistant to the stimulatory action of insulin on lipogenesis and that administration of A-4166 did not affect this response to insulin.

The tight control of glucose concentration in blood of patients suffering diabetes mellitus is very important in therapy because the persistent hyperglycemia results in an increased risk of the development of vascular complications (PAOLISSO et al. 1995l; LEROITH et al. 1996). In patients with non-insulin dependent diabetes mellitus (NIDDM) the control of glycemia is still suboptimal due to the difficulties in achievement of correct dose and timing of insulin administration as well as inconvenience of repeated insulin injections (PAOLISSO et al. 1995). To avoid the chronic hyperinsulinemia and the frequent insulin injections the oral hypoglycemic drugs with a rapid onset and short duration of action were developed. The response of insulin to a nutritional load in healthy human subjects consists of a rapid and transient increase of plasma insulin levels. The new d-phenylalanine derivatives (SDS DJN 608, A-4166)

stimulate the early insulin release which is important for the regulation of glycemia after the glucose load and meal intake (Sato et al. 1991). Thus, the peroral administration of A-4166 induces hyperinsulinemia which is similar to natural response of plasma insulin to a nutritional load. Several experimental studies in mice, rats and dogs demonstrated that SDS DJN 608 and A-4166 have been found to stimulate insulin secretion with rapid onset and short duration of action (Shinkai and Sato 1990; Fujitani and Yada 1994). Such early insulin response results in a decrease of glycemia and improvement of glucose tolerance.

Previous studies performed to evaluate the extrapancreatic effects of A-4166 or increased insulin levels on peripheral tissue metabolism showed an elevation of glucose transport in adipocytes from rats fed basal diet treated with A-4166. Small increase of GLUT 4 protein was found in adipocytes of rats fed basal diet, while a highly significant increase of GLUT 4 was found in those fed high fat diet. The administration of A-4166 normalized the lowered glucose oxidation in soleus muscle of rats fed high fat diet (Macho et al. 1999). These results indicate that the administration of A-4166 could result in the changes of glucose metabolism in muscle tissues and in adipocytes.

It was repeatedly demonstrated that insulin shows an inhibitory effect on lipolysis (Jacobsson and Smith 1972; Strade and Eaton 1977; Arner et al. 1981; Pedersen et al. 1982; Mills 1999) and stimulatory action on lipogenesis in adipose tissue (Macho et al. 1977). The aim of the present experiment was to study the effect of the novel hypoglycemic agent A-4166 on lipolysis and lipogenesis in adipocytes from normal rats fed standard laboratory chow or high fat diet and also in non obese animal strain with endogenous hypertriglyceridemia, insulin resistance and hypertension (HTG rats) bred by Vrana and Kazdova (1990) and, Klimes et al. 1995).

Materials and Methods

Animals. Three month old adult male Wistar rats of SPF colony (Anlab, Prague, Czech Republic) were divided into 4 groups of 10 animals each: 1. fed standard laboratory chow (basal diet, WBA) plus A-4166; 2. fed basal diet with vehiculum used for A-4166 (WB); 3. fed high fat diet plus A-4166 (WHFA); 4. fed high fat diet plus vehiculum (WHF).

The other 4 groups consisted of HTG animals from the Institute rat colony (aged three months): 5. fed basal diet plus A-4166 (HTGBA); 6. fed basal diet plus vehiculum, (HTGB); 7. fed high fat diet plus A-4166 (HTGHFA); 8. fed high fat diet plus vehiculum (HTGHF). The detailed composition of diets was described previously by Klimes et al. (1998).

The animals were housed by five in mesh wire cages located in temperature and light controlled rooms and fed *ad libitum* the above described diets for 3 weeks. The animals were not fasting before the experiment. The active substance (A-4166, 100 mg per kg b.w) or vehiculum of the same volume were administered by gavage 30 minutes before decapitation, after which the blood was withdrawn into ice cooled tubes and serum was separated for analysis of insulinemia (Rat Insulin Ria Kit, Linco Res. INC, St.Charles, MO, USA), glycemia (GLU, Boehringer, Mannheim, Germany) and free fatty acids (NEFA colorimetric kit, Randox Ardmore, UK), all anayses being performed with the use of BM Hitachi 704 analyzer.

The epididymal fat pads were rapidly removed and the adipocytes were isolated according to Rodbell (1960).

Lipogenesis. Aliquots of 10 % suspension of isolated fat cells in Krebs-Ringer bicarbonate solution (0.9 ml, KRBi) containing 5.88 mmol/l of glucose and 2 % of bovine serum albumin, were incubated two hours with increasing doses of insulin (10^{-9} and 10^{-7} mol/l added in 0.05 ml of KRBi) and with ^{14}C-U-glucose (7.4 kBq per sample, Amersham, UK) added in 0.05 ml of KRBi. Thereafter the lipids were extracted 3 times with 5 ml of chloroform:methanol (3:1) according to Folch et al. (1957). The lipid extract was evaporated, weighed and dissolved in 0.5 ml of chloroform:methanol and two portions of 0.2 ml were used for liquid scintillation counting and determination of the radioactivity of ^{14}C from labeled glucose incorporated into lipids. The incorporation of glucose into lipids was expressed in nmol of glucose per mg of lipids or per 10^5 cells.

Lipolysis. The lipolytic activity of adipocytes was estimated by incubation of 20 % suspension of fat cells in Krebs-Ringer bicarbonate solution containing 4 % bovine serum albumin and 12.5 mmol/l HEPES in the absence (basal lipolysis) or in the pres-

Table 1. Body mass, serum levels of insulin, glucose and fatty acids (NEFA) in Wistar (W) and HTG rats fed basal (BD) or high fat diet (HF) after the administration of A-4166 (A).

	BODY MASS g		INSULIN μU/ml		GLUCOSE mmol/l		NEFA mmol/ l	
	BD	HF	BD	HF	BD	HF	BD	HF
WISTAR								
O	438±15	454±20	82±10	130±16§	8.1±0.1	9.2±0.2§	0.36±0.03	0.54±0.04§
A	446±15	432±14	148±25*	158±17	5.1±0.4*	5.3±0.3*	0.28±0.02	0.37±0.02*
HTG								
O	296±13	328±10	42±4	75±6§	8.4±0.2	8.8±0.3	0.47±0.03	0.44±0.01
A	315±8	323±6	64±9*	87±7§	6.3±0.3*	5.1±0.3*	0.43±0.03	0.34±0.02*

§= p <0.05 for BD to HF, *= p <0.05 for **O** to **A**. **O** without and **A** after A-4166 treatment,

ence of norepinephrine (final concentrations 10^{-7} and 10^{-5} mol/l; Isoproterenol, Sigma, USA) for one hour at 37 °C. At the end of incubation 1.25 ml of 3.3 % KOH in ethanol and 2.5 ml of $MgSO_4$ were added, the suspension of cells was stired and centrifuged 10 min at 2500 rpm. The supernatant was used for glycerol analysis (Glycerol UV method, Boehringer, Mannheim, Germany). From the suspension of adipocytes 0.450 ml was used for the extraction and determination of lipids. The amount of glycerol released during the incubation was expressed per 10^5 cells or per 100 mg of lipids.

Statistical evaluation. The differences between two groups were tested using Student's t-test. Comparison between more than two groups were done by analysis of variance (ANOVA) followed by the Fisher least significance differences test.

Results

Body mass. The mean body mass of 3 month old Wistar Charles River rats at the end of experiment are shown in Tab. 1. There were no significant differences in the body mass increase of rats fed either basal or high fat diet for 3 weeks (e.g. 104±8 g in WB and 107±7g in WHF). However, the initial body mass of HTG rats (272±10 g) was significantly lower than that of Wistar rats of the same age (334±15 g; P<0.005). In HTG rats the increase of body mass after three weeks of feeding either basal or high fat diet was significantly less than in the appropriate Wistar controls, e.g only 24±4 g in HTGB (P<0.001) and 41±4 g in HTGHF (P<0.001), that in HTGHF animals being slightly higher than in HTGB group.

Insulinemia. The levels of insulin in serum were somewhat higher in non-fasting Wistar rats (Tab. 1) as observed previously in animals fasted at least 16 hours before the experiment (Macho and Fickova 1992), the values in those fed high fat diet being higher than in those fed basal diet. The administration of A-4166 resulted in significant increase of serum insulin concentration in Wistar rats fed basal diet. The increase of insulin levels was observed also in Wistar rats fed high fat diet, but this was not significant due to the great variation of final insulin levels and also of high basal insulin levels in animals fed high fat diet (Tab. 1).

In non-fasting HTG rats the serum insulin levels were lower than in the appropriate Wistar rats. The intake of high fat diet during three weeks was followed by the increase of insulin in serum. The administration of A-4166 caused a significant increase of insulin levels in HTG rats fed basal or high fat diet (Table 1).

Glycemia. Significant increase of serum glucose level was observed in both Wistar and HTG rats after feeding high fat diet (Tab. 1). The administration of A-4166 caused a highly significant diminution of glycemia in Wistar and HTG rats on basal and high fat diet. These results are in full agreement with previous observations on plasma glucose reducing effect of the A-4166 preparation.

NEFA. The serum levels of nonesterified fatty acids (NEFA) were increased in Wistar rats fed high fat

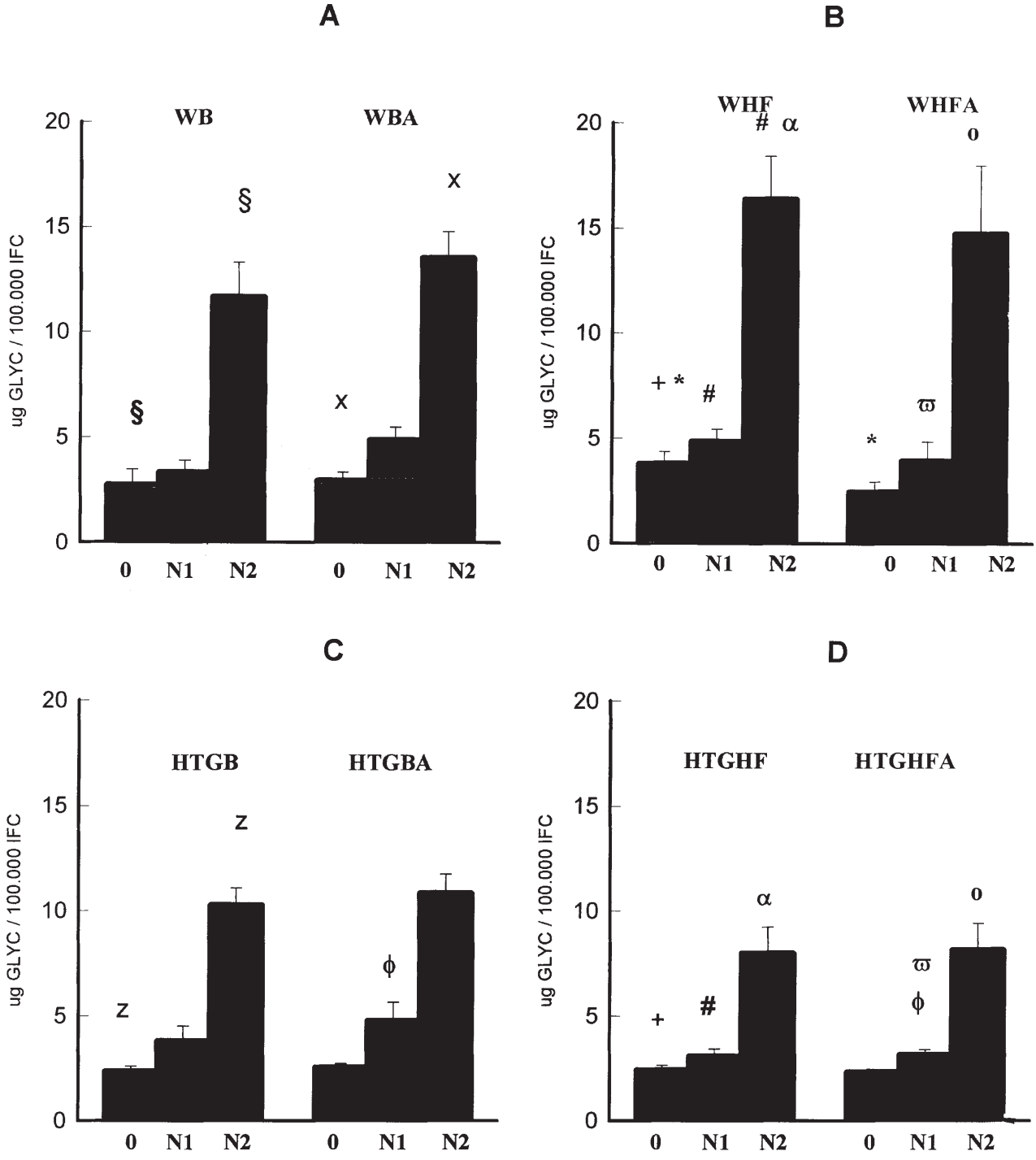

FIG. 1. Basal and norepinephrine stimulated lipolysis in rat adipocytes. A- Wistar rats on basal diet without (WB) and after (WBA) the administration of A-4166. B – Wistar rats on high fat diet without (WHF) and after A-4166 (WHFA). C- HTGB – HTG rats fed basal diet, HTGBA – HTG on basal diet and administration of A-4166. D- HTGHF and HTGHFA – HTG animals on high fat diet without and with administration of A-4166. 0- basal lipolysis, N1 – isoproterenol 10^{-7} and N2 – isoproterenol 10^{-5} mol/l, GLYC – glycerol released per 10^5 fat cells. The statistical significance of the groups with the same symbols are significant at level $p< 0.05$

diet (WHF group), but significant decrease of NEFA in serum was found in the appropriate group (WHFA) 30 min after the administration of A-4166 (Table 1). Significant increase of NEFA levels in serum was found in HTG rats on basal diet in comparison to Wistar animals, but the feeding of high fat diet did not affect the NEFA levels in HTG rats. No significant changes of serum NEFA concentration after the administration of A-4166 were found in HTG rats fed basal diet, but in HTG rats fed high fat diet the administration of A-4166 was followed by decrease of serum NEFA levels (Table 1). These results demonstrated that A-4166, besides the serum levels of insulin and glucose, shows also an effect on serum NEFA concentration especially in animals fed high fat diet.

Lipolysis. The basal lipolytic activity expressed per 10^5 cells was similar in Wistar control rats and in animals treated with A-4166 (Fig. 1A). The higher dose of norepinephrine was followed by more than 300 % increase of lipolytic activity and such stimulation of lipolysis was similar in control and A-4166 treated animals. The basal lipolytic activity of adipocytes from Wistar rats fed high fat diet was not significantly different from the values observed in control rats. However, the basal lipolysis of animals fed high fat diet and treated with A-4166 was lower as compared to nontreated group (Fig. 1B). The highly significant increase of lipolysis (300-400 %) was found after the addition of higher dose of norepinephrine also in rats fed high fat diet. The administration of A-4166 did not affect the stimulatory action of norepinephrine on lipolysis in Wistar rats fed high fat diet (Fig. 1B).

The basal lipolytic activity expressed per number of cells was similar in HTG rats as in Wistar rats on basal diet. However, the basal unstimulated lipolysis was significantly lower in HTG animals fed high fat diet as compared to Wistar rats on the same fat diet (Fig. 1B, 1D). The stimulation of lipolysis by norepinephrine was lower in HTG rats fed high fat diet in comparison to Wistar rats (Fig. 1B, 1D). There were no significant effects of A-4166 on basal and stimulated lipolysis in HTG animals.

The lipolytic activity was also expressed per lipid content in adipose tissue (data not shown). No significant differences in basal and stimulated lipolysis were found in Wistar rats fed basal or high fat diet and in HTG rats fed basal diet. Similarly as per number of cells the marked decrease of the stimulation of lipolysis by norepinephrine was noted in HTG rats fed high fat diet when compared to Wistar rats on the same diet or with HTG animals on basal diet.

Lipogenesis. The incubation of isolated adipocytes with radiocarbon labeled glucose results in incorporation of ^{14}C from glucose into lipids mainly into triglycerides (Fickova and Macho 1983). The addition of insulin stimulated the lipogenesis (as expressed per 10^5 cells) and significant increase was found with higher dose of insulin in Wistar rats on basal diet (Fig. 2a). The administration of A-4166 did not affect the basal lipogenesis and did not significantly change the effect of insulin added *in vitro*. The feeding of Wistar rats with high fat diet resulted in the slight increase of basal lipogenesis (Fig. 2A, 2B). The addition of insulin in vitro failed to have any significant stimulatory effect on lipogenesis in animals fed high fat diet suggesting the development of the resistance to insulin in high fat diet fed rats. The administration of A-4166 did not affect the lipogenesis in Wistar high fat diet fed rats. (Fig. 3B).

The rate of lipogenesis from glucose in HTG animals fed basal diet was similar as in Wistar control rats (Fig. 2A, 2C). However, the stimulation by insulin was slightly decreased as compared to Wistar rats. A significant decrease of basal lipogenesis in HTG rats fed high fat diet was noted when it was compared to Wistar rats on the same diet. Also in HTG animals fed high fat diet no significant stimulation of lipogenesis by insulin added *in vitro* was observed suggesting that these animals were insulin resistant. No significant differences in the rate of lipogenesis were observed in HTG rats with or without administration of A-4166. (Fig. 2C, 2D). Similar results were observed when the incorporation of glucose into lipids was expressed per mg of lipids (data not shown).

Discussion

Our findings showed that, in agreement with previous observations (Sato et al. 1991), the administration of A-4166 results in elevation of blood insulin levels and diminution of glycemia. These changes were observed not only in Wistar control rats but also in HTG animals and in hyperglycemic and hyperinsulinemic (insulin resistant) rats fed by high fat diet. Besides these effects the administration of A-

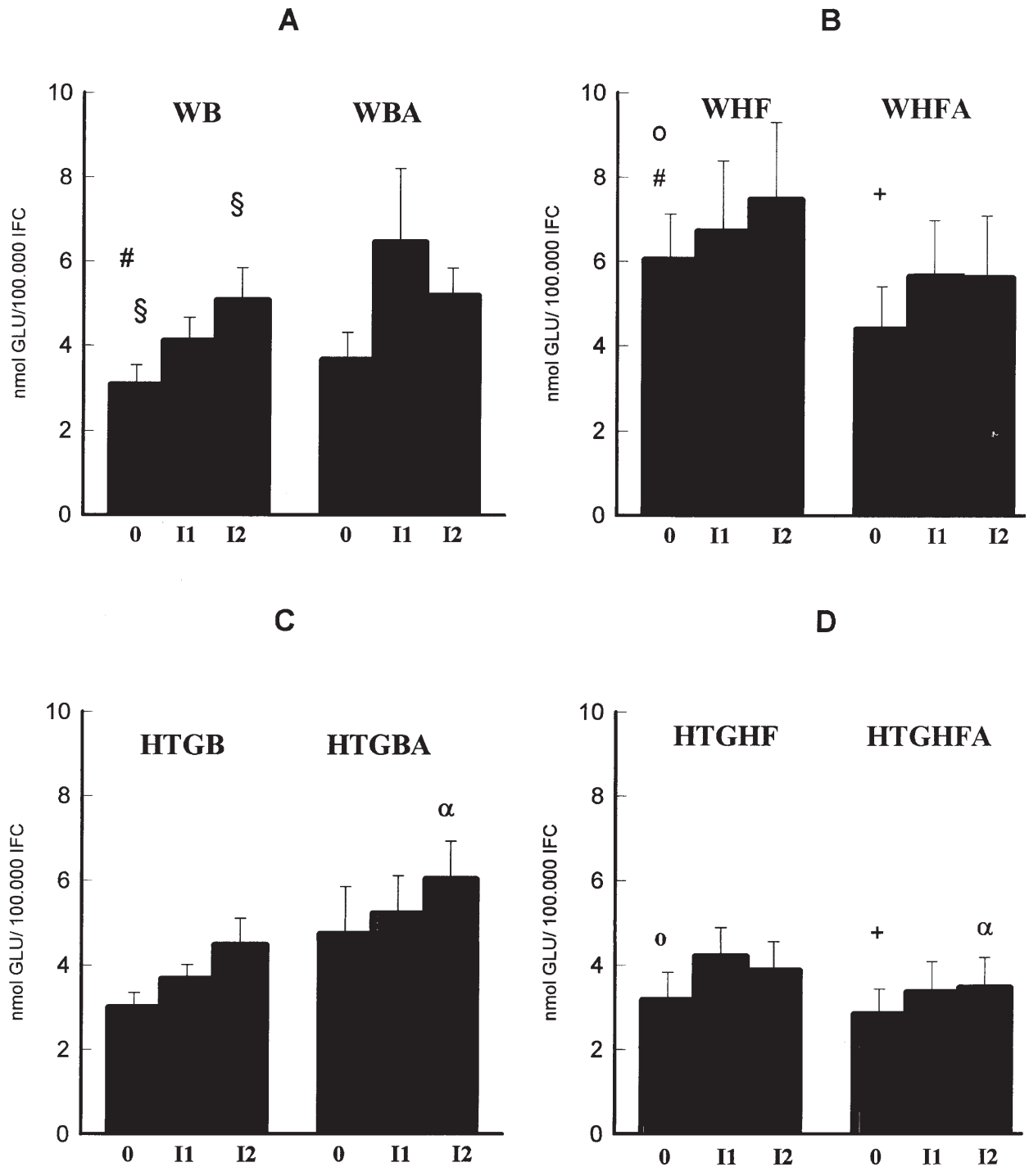

FIG. 2 Basal and insulin stimulated lipogenesis in adipocytes from Wistar (W) and HTG rats. WB, WBA – Wistar rats fed basal diet without and with A-4166. WHF and WHFA – Wistar rats fed high fat diet, AHFA with A-4166. HTGB and HTGBA – HTG animals on basal diet, HTGHF and HTGHFA – rats of HTG colony fed high fat diet. Incorporation of glucose in nmol/ 10^5 fat cells. The groups with the same symbols are statistically significant at level $p< 0.05$

4166 was followed by a decrease of NEFA concentration in serum of Wistar control rats (fed either basal or high fat diet) and HTG animals fed high fat diet. Lower levels of NEFA were also observed during an oral glucose tolerance test after the administration of A-4166 (Klimes et al. 1998). Serum levels of triglycerides were diminished after A-4166 administration in Wistar control rats, but no effect on serum triglycerides was noted in HTG animals (Macho et al. 1998). These results demonstrated that A-4166 has also an effect on lipid metabolism probably related to its insulinotropic action.

The studies of the lipolytic activity in adipocytes showed that the administration of A-4166 had no significant effect on basal and norepinephrine stimulated lipolysis in Wistar control and HTG animals fed basal diet. It was noted, however, that the basal lipolytic activity in Wistar rats fed high fat diet and treated with A-4166 was lower as compared to non treated animals on the same diet which suggests that A-4166 could increase the antilipolytic effect of insulin in insulin resistance induced by high fat diet. The stimulation of lipolysis by norepinephrine in adipocytes from Wistar rats fed high fat diet was not affected by A-4166 treatment.

The basal lipolysis was not affected by A-4166 in other type of insulin resistant rats, the HTG rats fed high fat diet. However, these HTG rats showed the low lipolytic activity even without the treatment with A-4166. It was interesting that lipolytic stimulatory action of norepinephrine was lower in HTG rats fed high fat diet as compared to Wistar animals on the same diet and the administration of A-4166 did not affect this change in the sensitivity of adipocytes to norepinephrine. These results suggest that the administrations of A-4166 can affect a basal lipolysis in adipocytes from Wistar rats fed high fat diet. A rapid and transient hyperinsulinemia observed also in other groups after the administration of A-4166 was not followed by decrease in basal lipolysis in spite of well known antilipolytic effects of insulin observed *in vivo* (Howard et al. 1984; Jensen et al. 1989; Hickner et al. 1999) or *in vitro* conditions in fat tissues (Castan et al. 1999; Kandulska et al. 1999). However, no direct evidence, such as turnover studies, was presented in these *in vivo* studies showing that the insulin induced plasma free fatty acid declines were a result of inhibition of adipose tissue lipolysis.

Furthermore, it was demonstrated that lipolysis of muscular lipids is also regulated by insulin. Jacob et al. (1999) showed by using microdialysis technique that glycerol release from muscular lipids is suppressed by insulin. Therefore it is possible that A-4166 induced FFA decline in serum levels could be a result of the changes in intramuscular lipolysis or increased utilization of FFA in peripheral tissues. The further studies are necessary to demonstrate the effects of A-4166 on metabolism of FFA, especially in muscle tissues.

The determination of lipogenesis in isolated adipocytes showed, that the administration of A-4166 did not affect the basal lipogenesis and did not significantly change the effect of insulin added in vitro. Further it was observed that the animals fed high fat diet failed to respond significantly to insulin and administration of A-4166 did not influenced this resistance of adipocytes to insulin.

The differences in the rate of basal lipogenesis were found in HTG rats fed high fat diet. The incorporation of glucose into lipids was increased in Wistar rats fed high fat diet, but in HTG animals no changes of lipogenesis were noted after feeding high fat diet. This finding is in agreement with lower augmentation of body mass and size of adipocytes of HTG rats fed high fat diet (Macho et al. 1998). The administration of A-4166 and the addition of insulin did not affect the rate of lipogenesis in HTG animals so the insulin resistance is present in these rats. The results of our studies showed that not only glucose utilization but also the processes of lipogenesis are insulin resistant in HTG animals.

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