Molecular and cellular actions of a structural domain of human growth hormone (AOD9401) on lipid metabolism in Zucker fatty rats

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ABSTRACT

A lipolytic domain (AOD9401) of human growth hormone (hGH) which resides in the carboxyl terminus of the molecule and contains the amino acid residues 177–191, has been synthesized using solid-phase peptide synthesis techniques. AOD9401 stimulated hormone-sensitive lipase and inhibited acetyl coenzyme A carboxylase (acetyl CoA carboxylase) in isolated rat adipose tissues, in a similar manner to the actions of the intact hGH molecule. The synthetic lipolytic domain mimicked the effect of the intact growth hormone on diacylglycerol release in adipocytes. Chronic treatment of obese

Zucker rats with AOD9401 for 20 days reduced the body weight gain of the animals, and the average cell size of the adipocytes of the treated animals decreased from 110 to 80 µm in diameter. Unlike hGH, synthetic AOD9401 did not induce insulin resistance or glucose intolerance in the laboratory animals after chronic treatment. The results suggest that AOD9401 has the potential to be developed into a therapeutic agent for the control of obesity.

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INTRODUCTION

A recent article on the history of growth hormone (Tattersall 1996) clearly reveals the fascination of the growth hormone (GH) molecule to numerous investigators over a long period of time. In the early 1960s, a working party of the Pituitary Hormone Committee of the UK Medical Research Council was established to initiate clinical trials of human (h) GH (Raben 1962). Pituitary hGH was used for the treatment of patients with short stature in the late 1960s until the early 1980s. The clinical applications of pituitary hGH were greatly reduced from the mid 1980s due to the possible transmission of the Creutzfeldt–Jacob disease (CJD). Meanwhile, the introduction of recombinant-hGH (Martial *et al.* 1979) has changed the whole field of hGH research and applications. hGH was the second

human hormone to be produced commercially using recombinant DNA technology. With large-scale production, the supply of hGH no longer restricts the types and number of human therapies. However, the complexity of the molecular mechanism of the hormonal actions is not yet fully understood. It is essential to have a better insight into its molecular actions for the efficient and safe applications to human use. This study focused on the lipolytic property of hGH and the potential role of the lipolytic fragment of hGH for the treatment of obesity.

A wide spectrum of cellular and metabolic activities of hGH has long been documented (Strobl & Thomas 1994). The hormone regulates somatic growth through hypertrophy and hyperplasia as a result of tissue differentiation and cell proliferation. Exogenous hGH also stimulates protein synthesis

and lipolysis and modulates carbohydrate metabolism and fluid-electrolyte balance. Our studies with genetically growth hormone-deficient mice also demonstrated the anti-lipogenic action of the exogenous hormone (Adamafio & Ng 1984). It has generally been recognized that hGH exerts a profound effect on body composition (Brummer & Bengtsson 1995). Systemic hGH administration reduces body fat mass as well as fat distribution (Rosenbaum *et al.* 1989, Bengtsson *et al.* 1992). The use of hGH for the treatment of human obesity has not been advocated due to serious side effects, including glucose intolerance, insulin resistance, sodium retention, hypertension, edema and carpal tunnel syndrome (Daughaday & Harvey 1994).

Human GH has a history as a lipolytic hormone dating back to the discovery by Lee and Schaeffer (1934) of decreased carcass lipid in rats treated with growth-promoting extract of anterior pituitary glands. However, the molecular mechanism of the lipolytic action of this multifunctional protein hormone is not fully elucidated at present. The concept that a large multifunctional protein might contain a number of discrete functional domains for a variety of specific biochemical or physiological actions (Paladini *et al.* 1979, Chretien 1999) led us to investigate the various functional domains of hGH. We first reported that the amino-terminus of the hGH molecule is the functional domain for the insulin-like action of the hormone (Ng 1990). The concept was further supported by the observations of Jeoung *et al.* (1993) and Lopez-Guajardo *et al.* (1998) that the sequence containing residues 108–129 might be the mitogenic domain of the hormone, and is consistent with the early observation of Liberti (1981) that the hormonal fragment 87–124 generated somatomedin-like activity. We have identified the metabolic domain responsible specifically for the lipolytic/anti-lipogenic activity of the hGH molecule. It resides in the hGH sequence containing the amino acid residues 177–191 and is referred to by us as AOD9401. Nuclear magnetic resonance (NMR) studies revealed that AOD9401 in solution retains a cyclic loop structure, similar to the conformational feature of the structural domain in the intact hGH molecule (Ogru *et al.* 2000).

Our preliminary studies showed that AOD9401 reduced by more than 20% the body weight gain of C57BL/6J (ob/ob) mice after intraperitoneal injections at a daily dose of 200 µg/kg body weight for 18 days (Natera *et al.* 1994). We now report the *in vitro* and *in vivo* activities of AOD9401 in fatty Zucker rats, demonstrate the molecular mechanism of the metabolic events and provide supporting evidence

for the development of AOD9401 or an analogue thereof as a specific and safe therapeutic agent for the treatment of human obesity.

MATERIALS AND METHODS

Preparation of synthetic peptide analogues

The hGH sequence containing the amino acid residues 177–191 (AOD9401) was prepared using solid-phase peptide synthesis methodology. N^α-9-fluorenylmethoxycarbonyl (Fmoc) chemistry was used for peptide synthesis. The peptides were purified with reversed-phase high performance liquid chromatography (RP-HPLC) and characterized for their purity, amino acid composition, and molecular weight with analytical RP-HPLC, amino acid analysis, and mass spectroscopy respectively.

Experimental animals

Obese Zucker (fa/fa) rats (Zucker & Zucker 1961) of both sexes, aged 15–16 weeks were fed on normal chow pellets (Clark King, Melbourne, Australia) consisting of 67% carbohydrate, 20% protein and 6% fat (the residue was crude fibres and minerals). Food and drinking water were available *ad libitum*. The animals were individually housed in plastic cages under the care of the animal technicians and were kept at an ambient temperature of 22 °C with a 12-h light/darkness cycle in the Department of Biochemistry and Molecular Biology, Monash University. The protocols for experiments on laboratory animals were approved by the Animal Ethics Committee of Monash University. The NHMRC Australian Code of Practice for the care and use of animals for scientific purposes (1997) was observed at all times.

Assays for acetyl-CoA carboxylase and hormone-sensitive lipase

The acetyl-CoA carboxylase activity was measured by determining the rate of carboxylation of acetyl-CoA with H[¹⁴C]O₃ into [¹⁴C]malonyl-CoA/[¹⁴C]bicarbonate (Ng & Heng 1988). Isolated adipocytes were preincubated at 37 °C for 30 min in a mixture of 50 mM Tris-HCl buffer (pH 7.5), 10 mM potassium citrate, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), and BSA (0.8 mg/ml). The reaction was then initiated by adding an aliquot of the cell suspension to 0.5 ml assay mixture containing also 3.75 mM ATP, 0.125 mM acetyl-CoA, and 12.5 mM NaH[¹⁴C]O₃ (final specific

activity 0.44 $\mu\text{Ci}/\mu\text{mol}$). After incubation at 37 °C for 10 min, the reaction was terminated with the addition of 0.1 ml 6 M HCl. The unreacted $\text{NaH}^{14}\text{C}\text{O}_3$ was removed and ^{14}C malonyl CoA was determined with a Wallac 1410 liquid scintillation counter. The specific activity of the enzyme was expressed as U/mg protein, where 1 U acetyl-CoA carboxylase was defined as that amount of enzyme which catalyzed the carboxylation of 1 nmole acetyl-CoA per min under the assay conditions described.

Hormone-sensitive lipase (HSL) activity of isolated adipocytes was evaluated by measuring the rate of hydrolysis of ^{14}C trioleic acid according to the method of Stralfors *et al.* (1987). The HSL activity of adipocytes was measured at 37 °C for 1 h in a final volume of 200 μl , containing 10 μmol phosphate buffer (pH 7.0), 15 μmol emulsified ^{14}C triolein, and 10^8 cells. The reaction was stopped by adding 1 ml of the fatty acid extraction mixture of chloroform-methanol-benzene (2:2:4, v/v/v) containing 50 μg oleic acid, followed by the addition of 67 μl 0.5 M NaOH. The ^{14}C radioactivity was measured with a Wallac 1410 liquid scintillation counter. The HSL activity was expressed as U/mg protein, where the release of 1 nmol of oleic acid per h was defined as 1 U enzyme activity. Cellular protein was determined with a modified method of Bradford (1976) using the commercially available BCA (bicinchoninic acid) Protein Assay Kit (#23225x; Pierce, Rockford, IL, USA).

Assays for free fatty acid, glycerol and diacylglycerol release

A colorimetric assay (Noma *et al.* 1973) was used for the determination of free fatty acid (FFA) from the incubation medium or plasma from animals. A 100 μl sample from incubation medium or plasma for FFA assay was placed in a 10 ml plastic centrifuge tube and then extracted by adding 3 ml organic solvents containing chloroform-heptane-methanol (49:49:2, v/v/v) and followed by 1 ml copper reagent. After mixing, the colour of the solution changed from yellow to greenish blue. The FFA concentration in the sample was determined by reading the absorbance at 610 nm from the standard curve of known FFA quantities (0–0.100 μmol), where palmitic acid (hexadecanoic acid) was used as the standard FFA.

Glycerol concentration in incubation medium or animal plasma was determined by an enzymatic method with glycerol kinase, pyruvic kinase and lactate dehydrogenase. Samples from incubation medium or blood plasma were placed into 10 ml

plastic centrifuge tubes and deproteinized with an equal volume of 10% trichloroacetic acid (TCA), followed by centrifugation for 8 min at 2600 rev/min (speed=5.5) at 4 °C using a DuPont Sorvall RT-6000 refrigerated centrifuge. After centrifugation, the solution in each sample was separated into two layers and the upper, clear supernatant was transferred into a 40 ml glass test tube with a glass stopper. The solution was then extracted twice with a fivefold excess of ether (20 ml $\times$ 2, 2 min) to remove TCA. The ether was then removed from the extract solution under vacuum overnight and the glycerol concentration was determined using the enzymatic method of McGowan *et al.* (1983).

Diacylglycerol (DAG) released into the incubation medium was determined with a radioenzymatic assay (Amersham, Castle Hill, NSW, Australia) employing *Eschericia coli* DAG kinase. Mixed micelle conditions were defined to solubilize DAG from the samples incubated with AOD9401. To each sample taken at different times, *E. coli* DAG kinase was used to start the assay and its quantitative conversion to ^{33}P phosphatidic acid in the presence of ^{33}P γ -ATP was allowed. A number of extraction steps were used to remove the unreacted ^{33}P γ -ATP. Separation of ^{33}P phosphatidic acid was achieved with the use of a 1 ml AmPrep minicolumn. The fractions containing the ^{33}P phosphatidic acid were collected, pooled and counted for radioactivity.

^{14}C Glucose incorporation into ^{14}C lipid

The rate of incorporation of exogenous ^{14}C glucose into total lipid in isolated adipose tissue was measured and used as an index of lipogenic activity (Salem & Wolff 1989, Harris *et al.* 1993, Natera *et al.* 1994). Tissues were preincubated in 2 ml Krebs–Ringer bicarbonate (KRB) buffer (pH 7.4) containing 40 mg defatted BSA and 0.2 mg glucose. Samples were placed in the incubator under an atmosphere of carbogen (95% O_2 /5% CO_2) for 1 h at 37 °C. After preincubation, each tissue was transferred to another flask which contained 2 ml fresh solution of the same composition, as well as ^{14}C glucose (final specific activity 0.05 $\mu\text{Ci}/\mu\text{mol}$), bovine insulin (0.1 mU/ml) and in the presence or absence of AOD9401, and was incubated for a further 90 min. Tissues were then removed from flasks and extracted with 5 ml chloroform-methanol (2:1, v/v). The ^{14}C lipid of each sample was measured with a Wallac 1410 liquid scintillation counter. The rate of total lipid synthesis was expressed as μmol ^{14}C glucose incorporated into lipid/mg tissue/min.

Determination of blood glucose

Blood samples (25 µl) from experimental animals were collected into heparin-coated tubes. The whole blood glucose level in each sample was measured immediately using a YSI Model-2300 STAT Glucose Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH, USA) with an immobilized glucose oxidase methodology.

Adipocyte isolation and cell size determination

Adipocytes were isolated from the epididymal fat pads of male Zucker fatty (fa/fa) rats (200–300 g) according to the method described by Roupas *et al.* (1990). The number of isolated adipocytes was estimated by counting an aliquot of a pre-determined volume of cells on a microscope slide, according to a modification of the method by Gliemann (1965). The isolated cells were suspended in KRB buffer prior to use for enzyme assays or cell size determinations.

Prior to cell size determination, the isolated adipocytes were fixed with formalin for 30 min. Centrifugation at 300 r.p.m. at 4 °C for 1 min was used to separate the formalin from the cells. This procedure allowed the cells to retain their original spherical shape. Aliquots of the fixed cells were placed onto microscope slides and the cell size of adipocytes was determined using a microscope (Nikon, Optiphot Biological Microscope, Tokyo, Japan). The image of the adipocytes was captured under constant illumination, teleconverted, amplified, digitized, and finally displayed on the colour monitor by the Microcomputer Imaging Device (MCID) system (Imaging Research Inc, St Catharines, Ontario, Canada) with M4 software (version 2.1 1996). The diameters of cell sizes were recorded and the mean and standard deviation were automatically measured in µm after being calibrated with standard length parameters.

Administration of AOD9401 to Zucker rats

AOD9401 was administered to the animals either intraperitoneally (Waynforth & Flecknell 1992) or intravenously. The intraperitoneal injection was administered to the lower left quadrant of the abdomen in the rat. The femoral vein and jugular vein were the two major sites used for the intravenous injection and clamp experiments with the rat under anaesthesia (Kraegen *et al.* 1983).

Slow-release AOD9401 tablets or the placebo formulated according to the method of Cardamone *et al.* (1998) were implanted under the skin layer on either side of the lower quadrant of the abdomen.

Mishel wound clips (9 mm. Mikron AUTOCLIP®; Becton Dickinson and Company, Sparks, MD, USA) were used to fasten the skin layer, and antibiotic powder (Cicatrín) was applied over the region in order to prevent infection. After surgery, the animals were placed in a cage, and regular inspection by the animal technician was made until consciousness was regained.

Determination of insulin sensitivity of Zucker rats using the clamp technique

For this preliminary long-term study, four Zucker rats were given daily an intraperitoneal injection of AOD9401 or saline. On the day of the clamp experiment, the animals were anaesthetized intraperitoneally with pentobarbitone (50 mg/kg body weight). An intravenous Silastic catheter (Dow Corning, 0.02 inch ID × 0.037 inch OD) was inserted into the right jugular vein for glucose infusion and a second catheter was put into the left carotid artery for blood sampling. During the experiment, blood samples were taken every 4 min from the carotid artery of each animal and their blood glucose levels were determined with an automated YSI glucose analyzer.

The basal level of blood glucose (G_0) was determined by averaging the readings of the samples taken at time 0, 4, 8, and 12 min. The *in vivo* experiment was then started by infusing the glucose-isotonic saline solution (30%, w/v) into each animal via the jugular vein using a peristaltic pump (Minipulse III) at a constant rate of 16.4 mg glucose/kg/min, according to previous studies in our laboratory (Jiang 1999). A stabilized steady-state blood glucose level (G_1) was achieved through the negative feedback of endogenous insulin. The G_1 was maintained for 20 min and the animal was then given isotonic saline with insulin (1 U/ml) by infusion via the jugular vein at a constant rate of 4.8 mU insulin/kg/min. The blood glucose level was again stabilized and regarded as the second steady-state glucose level (G_2) of the animal. The basal and the two steady-state blood glucose levels were used to determine the glucose utilization and insulin responsiveness of the animals. The difference in these levels between the two groups was used as physiological evidence to determine whether or not chronic treatment with AOD9401 affected carbohydrate metabolism by inducing insulin resistance.

Statistical analysis

All data are expressed as the mean ± S.E.M. The difference between two samples was analyzed with

the Student's *t*-test (Chase 1987). *P* values of <0.05 are accepted as statistically significant.

RESULTS

In vitro effects of AOD9401 on lipid metabolism in isolated adipocytes

In the initial studies of the *in vitro* effects of AOD9401 on lipid metabolism, its effect on the activities of the enzymes acetyl-CoA carboxylase and HSL in isolated adipocytes was examined. Table 1 indicates that both AOD9401 and hGH exerted similar effects and significantly reduced the acetyl-CoA carboxylase activity in a dose-dependent manner over the range of 0.1 to 10 µM of the tested substances. The basal level of acetyl-CoA carboxylase activity in rat adipocytes was 0.865 ± 0.025 U/mg protein. The maximum inhibitory effect on the enzyme activity was observed when cells were treated with 10 µM AOD9401 (0.544 ± 0.018 U/mg protein, about 37% reduction from the basal enzyme activity).

The activity of HSL was increased by AOD9401 or hGH over the range 0.1 to 10 µM (Table 1). The basal HSL activity in the rat adipocytes was 24.8 ± 0.4 U/mg protein. The increase in enzyme activity was dose-dependent with a maximum response, 32.3 ± 0.8 U/mg protein, at a dose of 10 µM AOD9401.

Biphasic release of diacylglycerol induced by hGH is well established (Roupas & Herington 1994). AOD9401 mimicked the effect of hGH on diacylglycerol release in adipocytes at the same molar concentration as hGH (0.45 nM). These data in Fig. 1 suggest that some similar signalling steps may be involved in mediating the lipid metabolic effects of AOD9401 and hGH.

In vitro effect of AOD9401 on adipose tissues

The acute *in vitro* effects of AOD9401 on lipid metabolism were further assessed at the tissue level by examining the rates of lipogenesis and lipolysis using isolated rat adipose tissue. The effect of AOD9401 on lipogenesis was determined by measuring the rate of incorporation of [14 C]glucose into lipid in isolated adipose tissues. Table 2 shows that AOD9401 at a concentration of 1 µM reduced the rate of lipogenesis in adipose tissue from the basal level, 4.05 ± 0.22 pmol/mg tissue/min, to 2.30 ± 0.15 pmol/mg tissue/min. At 10 µM, AOD9401 was able to achieve a 57% inhibition of lipid synthesis. This was consistent with its effect on acetyl-CoA carboxylase in which 43% reduction of enzyme activity was observed.

TABLE 1. *In vitro* effect of different concentrations of AOD9401 or hGH on the activity of acetyl-CoA carboxylase and HSL in the isolated rat adipocytes. Results are the means $\pm$ S.E.M. of 8 determinations

	Dose of AOD9401 (µM)				Dose of hGH (µM)			
	0	0.1	1.0	10.0	0	0.1	1.0	10.0
Acetyl-CoA carboxylase (U/mg protein)	0.865 ± 0.025	0.817 ± 0.015	0.657 ± 0.011	0.544 ± 0.018	0.865 ± 0.025	0.818 ± 0.020	0.691 ± 0.016	0.549 ± 0.014
HSL (U/mg/protein)	24.8 ± 0.4	28.3 ± 0.6	32.0 ± 0.4	32.3 ± 0.8	24.8 ± 0.4	28.8 ± 0.6	31.6 ± 0.4	31.8 ± 0.6

Significant differences ($P < 0.05$, Student's *t*-test) were observed in all AOD9401- and hGH-treated cells when compared with untreated control cells.

FIGURE 1. *In vitro* effect of AOD9401 on the production of a secondary messenger molecule, DAG. The time course study was conducted to measure the release of DAG from isolated adipocytes during incubation in the presence of 0.45 nM AOD9401. Results are means $\pm$ S.E.M. of 8 determinations. * $P < 0.05$ compared with the basal level at zero time (Student's *t*-test).

AOD9401 also exerted lipolytic effects on isolated adipose tissue in a dose-dependent manner over the range 0 to 1.0 μ M. The basal rate of glycerol release was 9.50 ± 0.50 pmol/mg tissue/min. In the presence of 0.1 μ M AOD9401, the glycerol level was increased to 13.17 ± 1.00 pmol/mg tissue/min and with 10 μ M AOD9401 it was increased to 15.00 ± 1.17 pmol/mg tissue/min. However, no further stimulation of lipolytic action was observed when the peptide concentration was increased over 10 μ M. The ratios of released FFAs to glycerol among samples in the test group were much less than 3, the theoretical value, and decreased when the concentration of AOD9401 was increased. These observations were attributed to the fact that some of the released FFAs might have been oxidized or re-esterified into triglycerides by the adipose tissue (Campbell *et al.* 1994).

***In vivo* effect of AOD9401 on adiposity of Zucker rats**

The body weights of obese Zucker rats implanted with slow-release AOD9401 at a dose of 450 μ g/kg body weight/day were monitored for 20 days. Results in Fig. 2 clearly indicate that a significant

effect of AOD9401 on the reduction of body weight gain became evident by day 8 and continued up to the end of the experiment on day 20. However, Zucker rats which received daily intraperitoneal injections of AOD9401 under similar experimental conditions usually had a 7–10% reduction in their body weight compared with the untreated control animals. This modulation of body weight gain was due, at least in part, to the reduction of body fat mass, as the *ex vivo* studies of the adipose tissues of the treated animals revealed the inhibition of the lipogenic activity by AOD9401 (Fig. 3). The average cell size of the adipocytes of the AOD9401-treated animals was reduced from approximately 110 to 80 μ m in diameter (Fig. 4). Fewer adipocytes with a diameter over 110 μ m were found in the treated animals than in the untreated obese controls (Fig. 5). These findings are consistent with previous observations in which the long-term intraperitoneal administration of AOD9401 reduced the adiposity of obese mice (Natera *et al.* 1994).

Acute effect of AOD9401 on basal blood glucose of Zucker rats

The basal blood glucose level of non-fasted Zucker fatty rats was 7.8 ± 0.8 mM. There was no significant change in circulating levels of blood glucose following a bolus intravenous injection of AOD9401 (250 ~ 1000 μ g/kg) to the animals (Table 3). Although animals showed a small acute response to AOD9401 at 1000 μ g/kg, the highest dose tested in this study, the hyperglycaemic effect of AOD9401 was only marginal (0.6 ± 0.3 vs 0.1 ± 0.2 mM, 30 min after administration) and quickly returned to basal levels within 3 h. These observations were different from those reported in previous studies (Ng & Bornstein 1978, Wade *et al.* 1982). The difference was probably due to the purity of the peptide preparations. However, the transient hyperglycaemic actions of hGH(177–191) peptide used in the earlier reports were also diminished within one hour.

Chronic effect of AOD9401 on glucose tolerance and insulin resistance

Glucose tolerance and insulin responsiveness of the treated Zucker rats were determined and compared with those of saline-treated controls, using the clamp technique (Coates *et al.* 1995, Morris *et al.* 1997). The results revealed that there were no significant differences in glucose tolerance or insulin responsiveness between the treated group and the control group of animals under the present experimental conditions (Table 4).

TABLE 2. *In vitro* effect of AOD9401 on the rates of lipogenesis and lipolysis in isolated rat adipose tissues. The tissues were incubated in the buffer without or with AOD9401 at different concentrations, and the rates of glucose incorporated into lipid (lipogenesis) and glycerol released from triglyceride (lipolysis) were measured. Results are the means $\pm$ S.E.M. of 12 determinations

	Dose of AOD9401 (μ M)			
	0	0.1	1	10
Rate of glucose incorporated into lipid (pmol/mg tissue/min)	4.05 $\pm$ 0.22	3.55 $\pm$ 0.15	2.30 $\pm$ 0.15	2.21 $\pm$ 0.18
Release of glycerol (pmol/mg tissue/min)	9.50 $\pm$ 0.50	13.17 $\pm$ 1.00	15.50 $\pm$ 0.67	15.00 $\pm$ 1.17

Significant differences ($P < 0.05$, Student's *t*-test) were observed between the presence and absence of AOD9401 in all cases.

FIGURE 2. Effect of chronic treatment with AOD9401 on cumulative body weight gain in Zucker (fa/fa) rats. Animals were treated by implantation of either placebo control (●) or AOD9401 (○) tablets at a dose of 450 μ g/kg body weight per day. Results are means $\pm$ S.E.M. of 4 animals in each group. * $P < 0.05$ compared with the control group (Student's *t*-test).

The basal blood glucose level (G_0) was calculated by averaging the first four blood samples taken over the 0 to 12th minute. The G_0 values after the 28-day treatment with saline or AOD9401 at a dose of 500 μ g/kg/day, were 8.3 ± 0.1 mmol/l (G_0) in the treated group and 10.4 ± 2.5 mmol/l (G_0) in the control group. The first steady-state blood glucose level (G_1) was calculated from the average of six blood samples taken from the 28th to 48th minute. This elevated blood glucose level, resulting from the infusion of exogenous glucose, is an *in vivo* physiological parameter of the animals which

FIGURE 3. *Ex vivo* effect of AOD9401 on the rate of lipid synthesis in adipose tissue isolated from animals after treatment as described in the legend to Fig. 2. Results are means $\pm$ S.E.M. of 12 determinations from 4 animals. * $P < 0.05$ compared with the control (placebo) group (Student's *t*-test).

directly relates to the degree of glucose tolerance. The treated animals received glucose at a rate of 4.10 ± 0.01 mg/min/kg body weight to maintain the glucose level (G_1) at 12.3 ± 0.5 mmol/l and the untreated animals were infused with a lower glucose concentration (4.01 ± 0.07 mg/min/kg body weight) and still had a higher G_1 glucose level (13.8 ± 0.7 mmol/l). No significant impairment in glucose utilization of the animals in the present experiment was found.

The second steady-state blood glucose level (G_2) was calculated by averaging six blood samples taken over the 84th to 104th minute. This reduced blood

FIGURE 4. Effect of AOD9401 on cell sizes of the adipocytes isolated from experimental animals treated either with AOD9401 implant or with placebo as described in Fig. 2. The cells were randomly selected and their cell diameters were measured. The results are means $\pm$ S.E.M. of 120 cell size determinations for each group of animals. * $P < 0.05$ compared with the control group (Student's *t*-test).

glucose level, resulting from the responsiveness to infused exogenous insulin, is an *in vivo* physiological parameter, which could be used to evaluate insulin sensitivity of the animals. Both groups of animals were given the same rate of insulin infusion (5.98 ± 0.1 mU/mg/kg). However, the final steady state level of blood glucose was $12.3 - 1.1 = 11.2$ mmol/l for treated animals, and was $13.8 - 1.2 = 12.6$ mmol/l for untreated controls. Thus the same amount of exogenous insulin removed more circulating glucose from the blood in treated animals than in untreated controls. The present evidence suggests that treatment with AOD9401 did not affect insulin sensitivity or glucose tolerance of the animals under the experimental conditions as described. Recent glucose clamp experiments with an analogue compound (AOD9604) produced the same result (Ng *et al.* 2000). The plasma insulin levels of the experimental animals had not been measured as no significant effect of the AOD compound on the endogenous insulin levels had previously been detected in our obese mice studies.

DISCUSSION

It is well recognized that hGH is a multifunctional protein molecule with well-established lipolytic/anti-lipogenic properties. We have recognized that discrete structural domains of the molecule may be responsible for specific metabolic functions of the hormone (Ng 1990). The present molecular studies of the lipolytic/anti-lipogenic domain (AOD9401) of the hGH molecule reveal that its *in vitro* actions in adipose tissues are identical to those of the intact hormone. The acute effects include the stimulation of HSL and inhibition of acetyl-CoA carboxylase in rat adipocytes. Consequently, an increase in lipolysis and a decrease in lipogenesis induced by AOD9401 in isolated segments of rat epididymal fat pads were observed. The lipolytic and anti-lipogenic actions of hGH have been well documented (Goodman & Grichting 1983, Davidson 1987, Brummer & Bengtsson 1995) although the precise molecular events involved in eliciting the metabolic changes remain largely unknown. Previous work from our laboratory has demonstrated that G proteins play a role in the longer-term effects of growth hormone, and protein kinase C is involved in the acute effects of the hormone (Roupas *et al.* 1991, Kelly *et al.* 1994, Roupas & Herington 1994). Treatment of rat adipocytes with hGH resulted in a transient and biphasic production of DAG which is present in signalling pathways involving both G proteins and protein kinase C. Protein kinase C is involved in mediating the lipolytic action of hGH (Gorin *et al.* 1990). It has been well documented that one GH molecule interacts with two receptors (Postel-Vinay & Kelly 1996), receptor dimerization provides the signal to generate the biological response of the target cells because it brings two Janus kinase (JAK2) molecules into close proximity, thereby facilitating transphosphorylation (Ymer *et al.* 1989). JAK2 phosphorylation leads to activation of a number of alternative signalling pathways. Activated JAK2 also induces the activity of MAP kinase and phospholipase C which results in hydrolysis of phosphatidylcholine to form diacylglycerol as a second messenger molecule (Rui *et al.* 1997). Interestingly, the small molecule, AOD9401, did not compete with 125 I-hGH for receptor binding in a competition assay (Hefferman *et al.* 2000), but was shown to stimulate DAG production in adipocytes in a similar manner to intact GH. Rowlinson *et al.* (1996) has reported that the metabolic activities of some GH fragments are not mediated through hGH receptor. It is likely that AOD interacts with a cellular receptor other than GH receptor but stimulates similar downstream signalling events as

FIGURE 5. Effect of AOD9401 on changes in cell size distribution of the adipocytes isolated from experimental animals after AOD9401 (open bars) or placebo (solid bars) implant as described in the legend to Fig. 2. A population of 120 cells was randomly selected and the distribution of their cell diameters was compared.

TABLE 3. *In vivo* effect of AOD9401 on blood glucose levels of Zucker fatty (fa/fa) rats. Animals were given different doses of AOD9401 i.v. Blood samples (40 µl/sample) were collected from the tail vein at the times indicated and whole blood glucose levels were measured immediately. Results are the changes (in nM) from the respective basal values and are shown as means $\pm$ S.E.M. of 4 animals in each group

	Dose of AOD9401 (µg/kg body weight)			
	0	250	500	1000
Time (min)				
0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
15	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3	0.2 $\pm$ 0.2	0.3 $\pm$ 0.2
30	0.1 $\pm$ 0.2	0.2 $\pm$ 0.2	0.4 $\pm$ 0.2	0.6 $\pm$ 0.3
60	0.1 $\pm$ 0.3	0.2 $\pm$ 0.3	0.2 $\pm$ 0.3	0.5 $\pm$ 0.3
120	0.2 $\pm$ 0.3	0.1 $\pm$ 0.3	0.2 $\pm$ 0.3	0.4 $\pm$ 0.3
180	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3	0.2 $\pm$ 0.4	0.3 $\pm$ 0.4

There were no significant differences between any treated group and the control group.

the GH molecule in the generation of second messenger DAG. The molecular mechanisms of the regulation of lipid metabolism for both GH and AOD require further investigation, although the present studies show that AOD9401 acts in an identical manner to GH, inducing DAG production in a similar biphasic pattern. The evidence so far is consistent with the concept that the carboxyl

terminal region of the molecule is the lipolytic/antilipogenic domain of hGH and is responsible specifically for the regulation of lipid metabolism of the hormone. Intact hGH stimulates hormone-sensitive lipase activity in adipocytes, thus decreasing the lipid content of adipose tissue (Dietz & Schwartz 1991). AOD9401, similar to the intact hGH molecule, activates the enzyme resulting in an

TABLE 4. Effect of chronic treatment with AOD9401 on glucose metabolism in Zucker (fa/fa) rats (360–370 g body weight). Animals were given a daily i.p. injection of AOD9401 at a dose of 500 µg/kg body weight (b.w.) per day for 28 days. After treatment, the glucose tolerance and insulin responsiveness of animals were determined using the clamp technique. Results are means ± S.E.M. of 4 animals in each group

Parameter	Treated	Control
Basal blood glucose level G_0 (mmol/l)	8.3 ± 0.1	10.4 ± 0.1
Rate of glucose infusion to achieve G_1 (mg/min/kg b.w.)	4.10 ± 0.01	4.01 ± 0.07
Difference of blood glucose level $G_1 - G_0$ (mmol/l)	4.0 ± 0.3	3.4 ± 0.1
Rate of insulin infusion to achieve G_2 (mU/min/kg b.w.)	5.98 ± 0.01	5.98 ± 0.01
Difference of blood glucose level $G_2 - G_1$ (mmol/l)	-1.1 ± 0.3	-1.2 ± 0.1

There were no significant differences between any treated group and the control group.

increase in the lipolytic activity in the adipocytes and subsequently a reduction in the fat cell size. Our previous studies have unequivocally demonstrated the reduction of body weight gain of young obese mice after 18 days treatment with AOD9401. Data suggested that the body weight changes were due to the reduction of fat mass (Natera *et al.* 1994). C57BL/6J ob/ob mice are known to have markedly enlarged adipocytes due to diminished lipase activity (Herberg & Coleman 1977). The present studies carried out with tissues from a different obese rodent model – fatty Zucker (fa/fa) rats – yield similar results. All these findings support the hypothesis that AOD9401 has no species specificity; it acts not only in obese mice but also in fatty rats.

The Zucker fatty rats implanted with slow-release tablets of AOD9401 were found to have reduced body weight gain as compared with the control animals over the 20-day experimental period. This significant reduction of body weight gain in the fatty rats is similar to our previously observed effect on obese mice under similar treatment with AOD9401 (Natera *et al.* 1994). For the present experiments, the discomfort and stress of the animals associated with daily injection were avoided. The present findings also reveal the feasibility of using different modes of AOD9401 administration for the same metabolic effect.

An increase in the lipogenic enzymes in adipose tissue of fatty Zucker (fa/fa) rats has been reported (Ellwood *et al.* 1985) and an elevation in lipogenesis in adipose tissues of young fatty rats is also generally known (Stern & Johnson 1977). Our *in vivo* data are consistent with the notion that the

elevated lipogenic activity in the treated fatty rats may be reduced by AOD9401. The *ex vivo* studies on the adipose tissues from the treated animals showed an inhibition of lipogenesis 16 h after implantation of AOD9401. The *ex vivo* evidence further supports the hypothesis that AOD9401 is the lipolytic/anti-lipogenic domain of the hGH molecule.

The diabetogenic effects of acute and chronic infusion of exogenous hGH are well known (Goodman & Grichting 1983, Daughaday & Harvey 1994) although the mechanism of these actions is not yet clear. Interestingly, the present study shows no change in insulin sensitivity in the fatty Zucker rats after daily intraperitoneal injection of AOD9401 at a dose of 500 µg/kg body weight for 28 days, as shown in the present glucose clamp experiments. The ability of the animals to respond to glucose load was not affected by the long-term treatment with AOD9401. The second steady-state blood glucose levels of animals, due to the effect of exogenous insulin infusion (5.98 mU/kg body weight/min) were also without statistical difference between the two groups. The results suggest that the difference in diabetogenic status between AOD9401 and the intact hGH molecule may be due to the fact that the diabetogenic domain may reside in a different portion of the intact hormone. This further supports the concept that the multifunctional hGH contains a number of distinct structural domains for a wide spectrum of metabolic actions.

In conclusion, the present results demonstrate that AOD9401 has significant effects on the regulation of lipid metabolism. Both acute and chronic

exposure of adipocytes, adipose tissue and animals to AOD9401 led to a shift 'downward' in lipid accumulation of the treated targets. A reduction in lipogenesis and a stimulation in lipolysis were observed. These alterations led to decreased rates of lipid storage in and increased rates of lipid mobilization from adipose tissue. These changes, in turn, led to the size reduction in adipocyte cell size and reduced body weight gain in the treated animals. On the other hand, results from the clamp experiments also revealed that AOD9401 did not induce any observable adverse effect on carbohydrate metabolism of the treated animals after chronic treatment. The findings in the present studies indicate that AOD9401 has the potential to be developed into a therapeutic agent for control of human obesity. On-going research to be reported elsewhere has produced evidence that human adipose tissues appear to be more responsive to the lipolytic/anti-lipogenic action of AOD9401 than that of porcine or rodent tissues *in vitro* (Heffernan *et al.* 1998).

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