Lipolytic and Hypolipidemic Properties of Newly Synthesized Aryloxypropanolamine Derivatives

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Abstract

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In this study, the lipolytic effect of two newly synthesized potential β_3-adrenergic agonists A482 and B496 in active acid forms was tested using isolated sliced epididymal adipose tissue of Wistar rats, and compared with Isoprenaline and BRL37344. Furthermore, effects of an eight-week oral administration of the newly synthesized substances on serum cholesterol, triglycerides, glucose, adiponectin, resistin and weight gain were studied in C57Bl/6J mice that were fed high energy diet.

The newly synthesized substance A482 (4-(2-{[2-hydroxy-3-(4-methyl-carbamoyl-phenoxy)propyl]amino}ethyl)phenoxy-acetic acid hydrochloride) was able to produce almost full lipolysis at a 1×10^{-7} M concentration, and its effect on the rat epididymal adipose tissue was similar to the specific β_3-adrenergic agonist BRL37344. Ethyl ester of this substance significantly lowered plasma total cholesterol ($p < 0.001$), resistin ($p < 0.01$), weight gain ($p < 0.01$), improved total/HDL-cholesterol ratio ($p < 0.01$) and increased circulating adiponectin ($p < 0.001$) in C57Bl/6J mice that were fed high energy diet. The second tested substance B496 did not show all activities expected from β_3-adrenergic agonists. Our results suggest that the newly synthesised substance A482 may represent a potent β_3-adrenergic agonist.

β_3-adrenoreceptor agonist, lipolysis, adiponectin, resistin, C57Bl/6J mouse

β_3-adrenergic agonists (β_3-AA) have been proposed as potential new drugs for the treatment of diabetes and/or obesity because of the hypoglycaemic and lipolytic effects found in some of these compounds. Moreover, their application in other therapeutic areas, such as hypercholesterolaemia and atherosclerosis, has been suggested (Zulet et al. 1999). β_3-Adrenergic receptor (β_3-AR) is a seven-transmembrane G-protein coupled receptor that activates the adenyl cyclase (Strosberg et al. 1997). Stimulation of this receptor produces a cAMP-dependent activation of lipase, greater production of uncoupling protein-1(UCP-1) in brown adipose tissue and higher insulin sensitivity. These activities result in the reduction of body weight and ameliorate diabetic symptoms in various animal models of obesity and diabetes (Kordik and Reitz 1999). β_3-AR mRNAs have also been detected in the brain, stomach, small and large intestines, gall and urinary bladders, and prostate. Human ventricular myocardium and atrium express low amounts of mRNA. In the gastrointestinal tract, the receptor controls smooth muscle relaxation, blood flow and gastric secretion (Berkowitz et al. 1995; Evans et al. 1996). Furthermore, it has been hypothesized that the β_3-adrenergic agonists reduce food intake via the central β_2- or β_3-adrenoceptor activation. A recent study supports this proposal. Food intake decreased when β_3-AA BRL-37344 was intraperitoneally injected into lean and Zucker rats (Tsujii and Bray 1998). β_3-AR in human ventricle mediates the negative inotropic effect (Guathier et al. 2000).

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After β_3-AR was cloned in 1989 (Emorine et al. 1989), a large number of β_3-AA was prepared and evaluated, and these fall under either the arylethanolamine or aryloxypropanolamine chemical families. The key success in this area is the discovery of selective β_3-AA with good oral bioavailability that lack cardiovascular or other effects mediated by the β_1- or β_2-adrenergic stimulation (Kordik and Reitz 1999). At the moment, the major beneficial effects have been shown on obesity and type 2 diabetes. Further clinical use is suggested for the treatment of frequent urination (Hu et al. 2001). β_3-AA also inhibit contractile activity of ileum and colon (Bond and Clarke 1988), and modulate neuronal bronchomotor inducing relaxation of airway smooth muscle (Martin and Advenier 1995).

The existence of β_3-AA in the adipose tissue and their involvement in the control of lipolysis was also investigated in the dog. Selective β_3-AA (BRL 37344, SR 58611A and CGP 12177) activated lipolysis in isolated dog adipocytes. Infused to conscious dogs, β_3-AA increased plasma non-esterified fatty acids (Galitzky et al. 1993). Chronic treatment with β_3-AA (ICI D7114) also led to decreased weight and abdominal girth in adult beagle dogs. It seems that weight loss was associated with increased energy expenditure, since there was no detectable effect of the drug on energy intake. The appearance of UCP-1 in adipose tissue of treated dogs may have resulted in increased oxygen consumption by this tissue (Champigny et al. 1991). These data suggest that β_3-AA could be a useful pharmacological tool in the treatment of dog obesity.

In this study we investigated the lipolytic effect of two newly synthesized potential β_3-AA agonists *in vitro* and hypolipidemic effect *in vivo*.

Materials and Methods

Lipolysis measurement

Male Wistar rats (250–300 g) originating in SPF breeds (Anlab, Prague) were housed in cages under standard conditions (temperature 20–24 °C, 12 : 12 light-dark cycle) and maintained on standard rodent chow (SPF M1) *ad libitum*. Upon arrival, the rats were quarantined for one week. The lipolysis measurement was conducted according to an earlier report (Lincová et al. 2002). The rats were fasted for 18 h and killed by decapitation; the epididymal fat pads were quickly removed and kept in saline at 37 °C. After drying on a piece of gauze, the fat pads were cut into small pieces, and samples (50–60 mg) were incubated at 37 °C, with shaking at 180 cpm (Environmental shaker - incubator Biosan ES-20) in 1 ml Krebs-Ringer bicarbonate buffer (pH = 7.4) containing bovine serum albumin (5 g/100ml) and glucose (0.18 g/100ml). The tested drugs, (--) Isoprenaline, BRL-37344 (Sigma), and the two newly synthesized substances, were added directly into the incubation medium at a concentration of 1×10^{-5} M and 1×10^{-7} M, and they were present in the medium for the whole incubation time. After 90 min the tissue and media were immediately cooled by placing the tubes in an ice bath. Then the tissue and media were instantly separated. Agonist-free cell suspensions were also incubated for the basal lipolysis rate measuring. The rate of lipolysis was determined by glycerol concentration in the incubation medium. The free glycerol reagent (Sigma) was used for a quantitative enzymatic determination of glycerol by spectrophotometry at 540 nm (spectrophotometer Thermo Spectronic Unicam UV 300). The glycerol release was adjusted to the tissue weight and is reported as μmoles glycerol released/g tissue 90 min.

Hypolipidemic study

Thirty-two 5-week-old C57Bl/6J male mice (purchased from the Medical Faculty of the Masaryk University Brno), initially weighing 19 ± 1 g, were housed in cages with free access to water and diet under controlled conditions (temperature 20–24 °C, 12 : 12 light-dark cycle). At first, all mice were raised on a normal diet for 7 days for adaptation. Then they were divided into four groups by controlled randomization so that the mean body weights in groups were not different. The first control group (control, n = 8) received a normal rodent diet (SPF M1), the second group (hyper, n = 8) was fed a special diet enriched with saturated fat (45% of energy) and simple saccharides (35% of energy) for inducing obesity and hyperlipidemic state. The third group (hyper + A, n = 8) was fed the same special enriched diet, which contained 10mg/kg diet of substance A482, and the last group (hyper + B, n = 8) received special diet plus 10mg/kg diet of substance B496. The body weight was measured every week and the weight gain was calculated. After eight weeks the mice fasted for 16 h were killed by decapitation. Blood was collected and serum was obtained by centrifugation at 1500 *g* for 15 min. Glucose from whole blood was analyzed by the glucose oxidase method (Glucose Analyzer Glucocard II), serum total and HDL cholesterol were determined by enzymatic colorimetric kits (BioVendor), triglycerides were determined by commercial kits (Lachema) using an automatic biochemical analyzer Advia 1650 (Bayer). Adiponectin and resistin were analyzed

by ELISA kits (BioVendor). The methodology of both studies was approved by Central Committee for Animal Protection and monitored by the Ethics Committee of the University of Veterinary and Pharmaceutical Sciences Brno.

Statistical Analyses

The results are expressed as means ± SEM and they were statistically evaluated using Unistat 5.1., Unistat Ltd., 1998. Comparisons between the groups were carried out using a one-way analysis of variance (one-way ANOVA). Differences indicated by the ANOVAs were analyzed using the Tukey-HSD test.

Results and Discussion

Lipolysis measurement

The rat epididymal fat pads are used for the *in vitro* investigation of the adipose tissue metabolism because they are easily accessible, and their shape consisting of several tips of adipose tissue at the epididymis enables preparation of approximately identical pieces without any further slicing (Faulhaber et al. 1972).

The study presented in this article examined the lipolytic responses of the sliced rat epididymal adipose tissue to mixed ß-agonist (--) Isoprenaline, selective $ß_3$-agonist BRL-37344 and two newly synthesized aryloxypropanolamines in de-esterified free acid form. Table 1 summarizes the effects of the tested drugs on lipolysis. The spontaneous glycerol release, i.e. basal lipolysis, was 1.84 µmol/g tissue/90 min. (--) Isoprenaline stimulated glycerol release to 4.5-fold (1×10^{-5} M) and 1.9-fold (1×10^{-7} M); BRL-37344 stimulated glycerol release to 4.0-fold (1×10^{-5} M) and 3.8-fold (1×10^{-7} M); the substance A482-free acid form (A-fa) stimulated glycerol release to 4.5-fold (1×10^{-5} M) and 4.0-fold (1×10^{-7} M) and the substance B496-free acid form (B-fa) 2.0-fold (1×10^{-5} M) and 1.9-fold (1×10^{-7} M). Adding of all drugs at higher concentrations (1×10^{-5} M) resulted in significant increase in glycerol concentration in the incubation medium. However, in case of lower concentration, there was a significant increase in glycerol concentration only caused by BRL-37344 and substance A-fa.

Table 1. Lipolytic effect of (--) Isoprenaline, BRL-37344 and the two newly synthesized substances on the sliced rat epididymal adipose tissue. Glycerol release was adjusted to the tissue weight and it is reported as µmol glycerol released/g tissue/90 min. Values are means ± SEM, n = 6 in all experiments.

	Concentration	
	1×10^{-5} M	1×10^{-7} M
(--) Isoprenaline	8.22 ± 1.23***	3.45 ± 0.88
BRL37344	7.34 ± 0.90***	7.05 ± 1.09***
A482-free acid	8.24 ± 0.96***	7.45 ± 1.10***
B496-free acid	3.65 ± 0.96*	3.47 ± 1.46
Basal lipolysis	1.84 ± 0.36	

* $p < 0.05$, *** $p < 0.001$ vs. basal lipolysis (Tukey-HSD test)

Both BRL-37344 and (--) Isoprenaline are full agonists and induce dose-dependent lipolysis on rat epididymal adipocytes (Fotovati et al. 2001). The lipolytic effect induced by Isoprenaline on sliced rat adipose tissue culminates at a concentration of 1×10^{-5} M (Lincová et al. 2002). However, β_3-AA are more potent and their lipolytic effect reaches a plateau at about 1×10^{-6} M (Fotovati et al. 2001). The newly synthesized substance A-fa was able to produce almost full lipolysis at a 1×10^{-7} M concentration and its effect on sliced rat epididymal adipose tissue was similar to the specific β_3-adrenergic agonist BRL37344. The second tested substance B-fa was not able to stimulate full lipolysis. We also tested lipolytic properties of new substances in ester forms (data not shown), which are believed to have better bioavailability. They produced lower concentration of glycerol in the incubation medium than acid forms.

Hypolipidemic study

A number of studies have shown that β_3-AA can be useful in the therapy of diabetes or obesity, and they have also been suggested to have a putative role in the treatment

Table 2. Effects of normal energy diet, hyper energy diet, hyper energy diet containing substance A482 and B496 (10mg/kg diet) fed for 8 weeks on plasma cholesterol, triglycerides, glucose, adiponectin, resistin and weight gain. Values are means ± SEM, eight mice C57Bl/6J in all groups.

Measurements	Groups in study			
	Normal	Hyper	Hyper + A	Hyper + B
Total cholesterol (mmol/l)	1.99 ± 0.07	3.86 ± 0.38†††	2.90 ± 0.36***	3.03 ± 0.27***
Total/HDL-cholesterol	1.13 ± 0.11	1.37 ± 0.19†††	1.19 ± 0.07 **	1.18 ± 0.07**
Triglycerides (mmol/l)	0.72 ± 0.12	0.49 ± 0.06†††	0.41 ± 0.06	0.45 ± 0.09
Glucose (mmol/l)	4.94 ± 0.91	6.50 ± 0.83††	5.41 ± 0.86	5.99 ± 0.76
Adiponectin (µg/ml)	27.57 ± 5.99	10.91 ± 1.75†††	20.16 ± 2.07***	11.3 ± 1.81
Resistin (ng/ml)	1.20 ± 0.26	1.47 ± 0.20	1.10 ± 0.15**	1.36 ± 0.21
Weight gain (g)	7.42 ± 0.13	7.96 ± 0.46	6.71 ± 0.51**	8.68 ± 0.10

†† $p < 0.01$, ††† $p < 0.001$ vs. normal group (Tukey-HSD test)
** $p < 0.01$, *** $p < 0.001$ vs. hyper group (Tukey-HSD test)

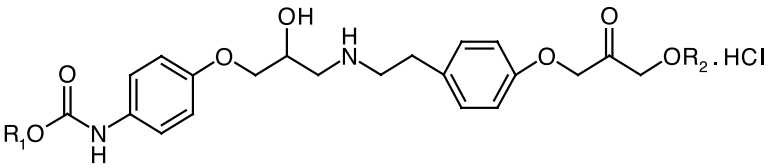

Fig. 1. Structure of the newly synthesized substances

Substance	R^1	R^2
A482	$-CH_3$	$-CH_2CH_3$
B496	$-CH_2CH_3$	$-CH_2CH_3$
A482-free acid	$-CH_3$	-COOH
B496-free acid	$-CH_2CH_3$	-COOH

of some lipid disturbances in animal models (Zulet et al. 1999). Various inbred strains of mice are differentially susceptible to developing obesity on high fat diets. One strain that is particularly vulnerable to this type of diet-induced obesity is the C57Bl/6J. When placed on a high fat diet, C57Bl/6J mice develop severe obesity, insulin resistance, and hyperglycaemia (Surwit et al. 2000).

In our experiment, the high energy feeding for eight weeks in C57Bl/6J mice led to a massive increase in total plasma cholesterol (194%, $p < 0.001$), a significant increase in plasma glucose (132%, $p < 0.01$), a significant decrease in total/HDL-cholesterol (82%, $p < 0.001$) and interestingly, triglycerides (68%, $p < 0.001$), which is consistent with a study published by Albers et al. (1999). The weight gain was higher (107%) in the hyper group but not significantly.

Further, we tested plasma levels of two newly discovered proteins secreted by the adipose tissue, which clearly plays a significant role in the pathogenesis of the hyperlipidemic state, insulin resistance and obesity. Adiponectin expression and serum levels are diminished in humans and animals with insulin resistance and obesity (Tsao et al. 2002). *In vivo*, adiponectin improves fatty acid utilization in liver and skeletal muscle of mice and reduces basal plasma glucose levels without affecting insulin and glucagon concentrations. Furthermore, it induces weight losses in mice without affecting their food intake (Fruebis et al. 2001).

Six years ago, the newly described protein resistin was proclaimed an important link between obesity and insulin resistance. In the original publication, serum resistin

concentrations were higher in mouse models of obesity (ob/ob, db/db, diet-induced obesity), and decreased with thiazolidinediones treatment (Steppan et al. 2001). Intravenous administration of resistin caused glucose intolerance and insulin resistance in mice. However, the excitement about the "resistin concept" slackened considerably when a year later Way et al. (2001) presented their evidence for the reduced expression of resistin in epididymal adipose tissue from four different murine models of obesity including ob/ob and db/db mice. In this study we found much lower adiponectin (40%, $p < 0.001$) and non-significantly higher resistin (123%) in the hyper group compared to the normal group.

Our study also showed that the newly synthesized substance A was a potent β_3-adrenergic agonist. It significantly lowered plasma total cholesterol (75%, $p < 0.001$), resistin (75%, $p < 0.01$), weight gain (84%, $p < 0.01$), improved total/HDL-cholesterol ratio (87%, $p < 0.01$), and increased circulating adiponectin (185%, $p < 0.001$) compared to the hyper group in this animal model of hypercholesterolaemia and hyperglycaemia. Plasma glucose (83%) and triglycerides (84%) levels tended to be lower but not significantly. Resistin levels and weight gain in mice treated with substance A were even lower than in mice on a normal diet.

The second tested substance was not as effective. It could improve only hypercholesterolaemia; other changes were not significant. Total cholesterol (78%, $p < 0.001$) and total/HDL-cholesterol (86%, $p < 0.01$) was decreased in the hyper + B group compared to the hyper group.

In conclusion, the newly synthesized substance A482 was a stimulator of lipolysis *in vitro* and it significantly lowered total cholesterol, resistin, weight gain, improved total/HDL-cholesterol ratio and increased circulating adiponectin *in vivo*. These data suggest that this substance may represent a potent rodent β_3-adrenergic agonist. β_3-adrenergic agonists could be useful in the treatment of obesity in the field of veterinary medicine, especially of the obesity of dogs.

Lipolytické a hypolipidemické vlastnosti nově syntetizovaných aryloxyaminopropanolů

Cílem této práce bylo zhodnotit lipolytický efekt dvou nově syntetizovaných potenciálních β_3-adrenergních agonistů A482 a B496 ve formě volných karboxylových kyselin na izolovanou epididymální tukovou tkáň potkanů kmene Wistar. Aktivita těchto látek byla porovnána s izoprenalinem a BRL37344. Dále jsme sledovali vliv osmitýdenního podávání těchto látek inbredním myším C57Bl/6J krmených vysoce energetickou dietou na sérové koncentrace cholesterolu, triacylglyceridů, glukózy, adiponectinu, resistinu a hmotnostní přírůstek.

Nově syntetizovaná látka A482 (hydrochlorid 4-{2-[(2-hydroxy-3-(4-metylkarbamoyl)fenoxypropyl)amino]ethyl}fenoxy octové kyseliny) v tomto pokusu do sáhla téměř maxi-mální lipolýzi již v koncentraci $1x10^{-7}$ M a její lipolytické působení na epididymální tukovou tkáň vykazovalo podobný charakter jako působení selektivního β_3-adrenergního agonisty BRL37344. Etyl ester této látky u myší C57Bl/6J signifikantně snižoval sérové hladiny celkového cholesterolu ($p < 0.001$), podílu celkového/HDL-cholesterolu ($p < 0.01$), resistinu ($p < 0.01$) a hmotnostního přírůstku ($p < 0.01$). Naopak hladiny cirkulujícího adiponectinu byly zvýšené ($p < 0.001$). Druhá z testovaných látek B496 pouze částečně prokázala aktivitu očekávanou u β_3-adrenergních agonistů. Výsledky této studie naznačují, že látka A482 může být účinným β_3-adrenergním agonistou.

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