A Novel Adrenaline Derivative, AZ002, and Its Hypoglycemic Action in Yellow KK Mice

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ABSTRACT—AZ002 (L-threo-(3,4-dihydroxy phenyl)-N-methyl serine methyl ester) is a newly synthesized adrenaline derivative. AZ002 caused relaxation of rat jejunum (β3-receptors) (ED50=18 μM), but did not affect the atrial rate (β1) or tracheal relaxation (β2) at a concentration of 0.3 mM. The pA2 values for propranolol in inhibiting the isoproterenol- and AZ002-stimulated relaxation of rat jejunum were 6.27 and 6.33, respectively. Thus, AZ002 is a selective agonist for β3-adrenoceptor. AZ002 stimulated lipolysis (ED50=10 μM) and glucose uptake (ED50=1 μM) in rat adipocytes. In both cases, stimulation was antagonized by high concentrations of the β-adrenoceptor antagonist propranolol, but not by the α-adrenoceptor antagonist phentolamine. The effect of AZ002 on glucose uptake was synergistic with that of insulin. AZ002 was also assessed in vivo by using genetically obese mice (KK/Ay strain) with hyperglycemia. Administration of AZ002 in the diet for a week decreased blood glucose and non-esterified fatty acids.

Keywords: AZ002, Adrenaline derivative, β3-Adrenoceptor, Adipocyte

Resistance to insulin-stimulated glucose uptake is present in the majority of patients with impaired glucose tolerance and non-insulin dependent diabetes mellitus (NIDDM) (1, 2). However, current oral therapy for NIDDM relies on the sulfonyl ureas and predominantly addresses the insulin-secretory defects rather than the insulin-sensitivity defects of this disease (3, 4). Therefore, research effort has been directed toward the discovery of drugs that will improve glucose uptake and hyperglycemia.

At the cellular level, impaired glucose uptake has been demonstrated in isolated adipocytes (5) and muscle strips (6, 7) from obese and diabetic patients. Therefore, we have screened compounds that stimulate glucose uptake in isolated rat adipocytes, because such compounds may represent an improved therapeutic approach to the treatment of NIDDM. Among the compounds synthesized in our laboratory, we found that AZ002 showed potent stimulation of glucose uptake and lipolysis.

Recently, it was demonstrated that the lipolytic adrenoceptor associated with thermogenesis in brown adipose tissue was of neither the β1- nor the β3-subtype (8). In addition, the existence of adrenoceptors distinct from classical α- and β-subtypes has been demonstrated in rat and guinea pig intestine (9). These were referred to as atypical β-adrenoceptors and termed the β3-receptor. β3-Adrenoceptor agonists stimulate lipolysis and thermogenesis by selectively increasing the energy expenditure within brown adipose tissue, thereby providing a potential pharmacological treatment for obesity and NIDDM (10).

In this report, we describe the agonistic activities of AZ002 on β-adrenoceptors and on the stimulation of glucose uptake and lipolysis in rat adipocytes. We also examined whether AZ002 improved hyperglycemia in a mouse model of NIDDM which is genetically obese and hyperglycemic (10, 11).

MATERIALS AND METHODS

Chemicals

Insulin (porcine pancreas), collagenase (type II), (-)isoproterenol hydrochloride, (±)adrenaline hydrochloride, D-propranolol and bovine serum albumin (Fraction V) were purchased from Sigma (St. Louis, MO, USA). D-[3-3H]Glucose (654.9 GBq/mmol) was from NEN (DuPont, Wilmington, DE, USA), and toluene scintillation cocktail was from Packard Instrument Company (Meriden, CT, USA). AZ002 (L-threo-(3,4-dihydroxy phenyl)-N-methyl serine methyl ester) and NB108 (L-threo-(3,4-dihydroxy phenyl)-N-methyl serine) (Fig. 1) were synthesized by our company (Banyu, Tsukuba). All other chemicals used were of the highest purity available.
Adipose cells were isolated from the epididymal fat pads of 200 to 250 g rats according to the method of Rodbell (12) with some modifications. Briefly, the fat pads were removed and digested with collagenase (3 mg/3 ml/g of tissue) for 30 min at 37°C. The resultant cell suspension was filtered through gauze, and the suspension was centrifuged (500 x g for 1 min) at room temperature. The floating cells were pooled and washed 4 times and diluted (1 ml cell pack/ml) with Krebs-Ringer buffer (composition: 130 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄ and 20 mM HEPES) and 3.5% defatted bovine serum albumin at pH 7.4.

Measurement of glucose uptake stimulation

The adipose cell suspension (about 10⁵ cells/ml) was stirred to maintain homogeneity and 800-μl aliquots were transferred to polyethylene tubes. After the addition of the test compounds, the tubes were preincubated for 5 min in the presence or absence of insulin. Reactions were started by adding 0.3 nCi of d-[3-³H]glucose to a final volume of 1 ml and incubated for a further 120 min. Reactions were terminated by the addition of 3 ml of toluene scintillation cocktail and vortexing to extract lipids into the organic scintillation phase. This method was originally developed for adipocytes by Moody et al. (13). In the presence of glucose at the low concentration used here, glucose uptake is rate-limiting for the conversion of glucose into lipid (14, 15). In this assay, there is a good linear relationship between the glucose uptake and incubation time (0–6 hr). Adenosine deaminase did not affect the glucose uptake activities.

Lipolysis

The lipolytic response was determined by measuring the amount of non-esterified fatty acids according to the method of Duncombe (16). A control response in the absence of agonists and a maximal response in the presence of isoproterenol were obtained for each experiment. The lipolytic responses are expressed as a percentage of the maximal lipolysis obtained with isoproterenol.

Rat jejunum

The mid-portion of the small intestine (jejunum) was removed from male SD rats (400–500 g) (9). After the intraluminal contents were flushed, longitudinal segments (±1.5 cm) were mounted in 20-ml organ baths under 0.5 g of tension in Krebs-Henseleit solution (composition: 118 mM NaCl, 5.6 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 1.3 mM NaH₂PO₄, 25 mM NaHCO₃ and 5.5 mM glucose) oxygenated with 95% O₂ / 5% CO₂ (pH = 7.4) and kept at 37°C. Responses were measured isotonically. After a steady baseline was obtained, the test compound was added cumulatively to the organ baths, until a maximal response was obtained. The response to the test compound is expressed as a percentage of the maximal relaxation response obtained with isoproterenol.

Guinea pig trachea

The trachea was removed from male Hartley guinea pigs (500–600 g). Tracheal transverse preparations were mounted in 5-ml organ baths under 0.6 g of tension in Krebs-Henseleit solution oxygenated with 95% O₂ / 5% CO₂ (pH = 7.4) and kept at 37°C. Responses were measured isometrically. After a steady contraction to 100 μM histamine was obtained, the test compound was added cumulatively to the organ baths, until a maximal response was obtained. The response to the test compound is expressed as a percentage of the maximal relaxation response obtained with isoproterenol.

Guinea pig right atrium

The right atrium was removed from male Hartley guinea pigs (500–600 g). The tissue was mounted in 20-ml organ baths under 1 g of tension in Krebs-Henseleit solution oxygenated with 95% O₂ / 5% CO₂ (pH = 7.4) and kept at 31°C. After reaching a steady baseline rate, the test compound was added cumulatively to the organ baths, until a maximal response was obtained. The response to the test compound is expressed as a percentage of the maximal rate obtained with isoproterenol.

Drug administration and blood sampling

Male yellow KK mice were purchased from Clea Japan.
(Tokyo). They were housed in individual cages and main-
tained on a moderately high-calorie laboratory diet, 
Oriental CMF (Oriental Yeast, Tokyo), and water ad 
labitum. They were used for experiments when their body 
weights were 40–46 g (4–5 months of age), and they had 
well-developed hyperglycemia (15–20 mM) and hyper-
insulinemia (10–12 nM) (17). Animals were given either 
powdered diet alone or diet mixed with AZ002 (30 
mg/100 g diet) for 7 days. For blood sampling, animals 
were fasted for 24 hr; then the tip of the tail vein was cut 
by a razor at 9–10 a.m., and blood was collected in 
heparinized hematocrit tubes. The plasma, separated 
by centrifugation, was stored in a refrigerator and measured 
for glucose and non-esterified fatty acids (NEFA) with 
commercial kits (Wako, Osaka). An oral glucose toler-
ance test (2 mg of glucose/g body weight) was also per-
formed. The data are expressed as means±S.D. or 
S.E.M. and analyzed by Mann-Whitney’s U-test.

RESULTS

Glucose uptake and lipolysis in the absence of insulin

The effects of AZ002, adrenaline, noradrenaline and 
and isoproterenol on glucose uptake and lipolysis were inves-
tigated in rat adipocyte suspensions (Fig. 2). AZ002, iso-
proterenol, noradrenaline and adrenaline, in the absence 
of insulin, induced dose-dependent increases in glucose 
uptake (Fig. 2A) and lipolysis (Fig. 2B). Bell-shaped 
dose-response curves were observed for stimulation of 
glucose uptake, but not for lipolysis. The ED50 values of 
isoproterenol, noradrenaline, adrenaline and AZ002 for 
glucose uptake were 15, 30 and 60 nM and 1 ,uM and for 
lipolysis, 50, 110 and 300 nM and 10 pM, respectively. 
The sensitivity (ED50) for glucose uptake was several 
times more potent than that for lipolysis with all com-
ponds. AZ002 stimulated lipolysis by 1.4-fold above the 
maximum response (Vmax) with isoproterenol, whereas 
noradrenaline and adrenaline showed a response similar 
to that of isoproterenol. NB108 (Fig. 1), which is the free 
acid of AZ002, did not stimulate either glucose uptake or 
lipolysis even at 1 mM (not shown). This indicates that a 
methyl ester moiety at the carboxyl group in AZ002 is 
essential to elicit adrenoceptor agonistic activity.

Glucose uptake and lipolysis in the presence of insulin

The effects of insulin on AZ002-induced glucose uptake 
and lipolysis were investigated in rat white adipocytes. 
The effect of AZ002 on glucose uptake was synergistic 
with that of insulin (Fig. 3A). In contrast, as expected, 
insulin significantly inhibited AZ002-stimulated lipolysis 
in a dose-dependent manner (Fig. 3B). These results indi-
cated that AZ002 predominantly stimulated glucose up-
take rather than lipolysis in the presence of a high con-
centration of insulin.

Effects of adrenoceptor antagonists on glucose uptake 
and lipolysis by AZ002

Phentolamine, an a-adrenoceptor antagonist, failed to 
antagonize glucose uptake or lipolysis. However, high 
concentrations of propranolol, a ß-adrenoceptor an-
tagonist, antagonized both glucose uptake and lipolysis in 
a dose-dependent manner (Fig. 4), indicating that AZ002
acts via the \( \beta \)-adrenergic receptor in stimulating both processes. It should be noted that propranolol showed different potencies in the inhibition of AZO02-stimulated glucose uptake (IC50 = 2.5 \( \mu \)M) and lipolysis (IC50 = 0.65 \( \mu \)M). Values represent means \( \pm \) S.E.M. of 3 experiments. Experiments in A and B were performed at the same time.

**Action mechanism of AZO02**

To investigate the mechanism of action of AZO02, the relaxation of rat jejunum (\( \beta_3 \)) (9) and guinea pig trachea (\( \beta_2 \)) (8), and the stimulation of guinea pig atrial rate (\( \beta_1 \)) (8) were investigated.

Relaxation of rat jejunum with AZO02, isoproterenol and the selective \( \beta_3 \)-adrenoceptor agonist BRL37344 (sodium-4-[2-hydroxy-2-(3-chlorophenyl)ethylamino] propyl phenoxyacetate) without precontraction is shown in Fig. 5. All agonists caused dose-dependent relaxation. From these dose-response curves, the values of ED50 (M) for BRL37344, isoproterenol and AZO02 were calculated as 1.7 \( \pm \) 0.2 \( \times \) 10^{-8}, 2.4 \( \pm \) 0.7 \( \times \) 10^{-7} and 1.8 \( \pm \) 0.4 \( \times \) 10^{-5}, respectively.
respectively. Isoproterenol and AZ002 showed full responses, whereas BRL37344 acted as a partial agonist (about 80% of the maximal isoproterenol response) with a relatively high potency. Propranolol caused parallel shifts to the right in the dose-response curves of isoproterenol and AZ002. The pA2 values (negative logarithm of the molar concentration of propranolol causing a twofold shift in dose-response curves) for isoproterenol- and AZ002-stimulated relaxation were 6.27 and 6.33, respectively. NB 108 showed no effect on relaxation of the jejunum at concentrations as high as 0.3 mM.

Isoproterenol and BRL37344 induced dose-dependent relaxation of tracheal smooth muscle preparations precontracted with 100 nM histamine. BRL37344 acted as a partial agonist (74% of maximal isoproterenol response). The values of ED50 (M) for isoproterenol and BRL37344 were 1.4±0.3×10^{-9} and 1.2±0.2×10^{-7}, respectively (Fig. 6A). In contrast, neither AZ002 nor NB108 showed relaxation of tracheal tissue at concentrations as high as 0.3 mM.

Isoproterenol and BRL37344 produced dose-dependent stimulatory effects on atrial rate. The values of ED50 (M) for isoproterenol and BRL37344 were 2.0±0.2×10^{-9} and 1.3±0.1×10^{-6}, respectively (Fig. 6B). In contrast, neither AZ002 nor NB108 affected the atrial rate at concentrations as high as 0.3 mM.

**Blood glucose and NEFA in diabetic mice**

Yellow KK mice showed moderate hyperglycemia (11.6±2.4 mM) and high NEFA levels (767.0±121.2 μEq/l) in the fasted state (means±S.D., n=7). AZ002 was administered at a dose of 45 mg/kg/day in the diet for 7 days. The daily dose was calculated from the food intake. Plasma glucose and NEFA were determined in the fasted state. AZ002 lowered both plasma glucose (values after 7 days, mM: control group, 10.9±0.5; AZ002-treated group, 7.6±1.0; Fig. 7A) and NEFA levels (values after 7 days, μEq/l: control group, 765.6±88.4; AZ002-treated
group, 477.7±72.9; Fig. 7B). As shown in Fig. 7A, AZ002 also produced some improvement in glucose tolerance. No significant differences in body weight or food intake were observed between the control and AZ002-treated groups. However, in accordance with improved hyperglycemia, water intake volume in the AZ002-treated group (127±4 ml/7 days) was moderately decreased compared to that in the control group (148±7 ml/7 days).

DISCUSSION

AZ002 showed β-adrenoceptor agonistic activity that was selective for the β3-receptor, resulting in lipolysis (Fig. 2) and jejunum relaxation (Fig. 5). This compound did not affect heart rate (β1) or bronchodilation (β2) at a concentration of 0.3 mM. AZ002 is less potent than the non-catecholamine agonist, BRL37344 (8) (Fig. 5), but as potent as SM11044 (L-threo-3-(3,4-dihydroxyphenyl)-N-[3-(4-fluorophenyl)propyl]serine pyrrolidine amide hydrobromide), a catecholamine derivative that stimulates lipolysis in rat white adipocytes (EC50=1.2×10⁻⁶ M) (18). SM11044 also stimulated relaxation of guinea pig trachea (EC50=1.3×10⁻⁷ M) and increasing right atrial rate (EC50=6.9×10⁻⁶ M) (18). Thus, so far as we know, AZ002 is the most selective catecholamine derivative that shows agonistic activity against the β3-subtype.

It was reported that the biological effects of BRL35134A, the parent methylester of BRL37344, were mediated by its free acid, BRL37344 (8). NB108, the free acid of AZ002, did not display β-adrenoceptor agonistic activity. Thus, unlike BRL35134A, AZ002 is not a prodrug. It is well-known that adrenaline, the mother compound of AZ002, shows agonistic activity against not only β3-adrenoceptors but also all subtypes of α- and β-adrenoceptors. Therefore, it should be noted that the simple modification of the α-carbon in the chemical structure of adrenaline, as in AZ002, induces selective agonistic activity for the β3-subtype. This finding may be useful for the study of molecular interactions between catecholamines and their receptors.

Only the catecholamines are full agonists for human adipocytes, whereas for rat adipocytes, both catecholamines and non-catecholamine β-adrenoceptor agonists have full agonistic action (19, 20). Therefore, AZ002 is expected to show a higher level of intrinsic activity than non-catecholamine agonists in human adipocytes since it is a catecholamine derivative.

In rat adipocytes, isoproterenol, noradrenaline, adrenaline and AZ002 stimulated glucose uptake and lipolysis with the same order of potency. This suggests that the stimulation of glucose uptake and lipolysis are functionally linked, at least at low agonist concentrations. Indeed, catecholamines were less effective for stimulating glucose uptake when used at high concentrations, resulting in bell-shaped dose-response curves. In contrast, the bell-shaped curves were not seen for stimulation of lipolysis. Such a relationship was also demonstrated for stimulation of glucose uptake (bell-shaped curves) and respiration (non bell-shaped curves) in rat
brown adipocytes by noradrenaline, dibutyryl cAMP, 3-isobutyl-1-methylxanthine and palmitic acid (21).

Transport of glucose into cells is accomplished by a membrane-associated carrier protein, specific glucose transporter. Five functional glucose transporter isoforms have been identified that have distinct tissue distributions and biochemical properties (22–24). Adipocytes predominantly contain the glucose transporter, GLUT4 isoform, which is responsible for insulin-stimulated uptake of glucose in these tissues. This action of insulin is accomplished via a translocation of GLUT4 transporters from low density microsomes to the plasma membrane.

In this report, we demonstrated the stimulating effect of AZO02 on glucose uptake and found that the action of AZO02 was synergistic with that of insulin (Fig. 3). This would indicate that part of the stimulating effect of AZO02 on glucose uptake could be related to the pathway of insulin action. Indeed, cAMP regulates translocation of insulin-sensitive GLUT4 (25).

We administered AZO02 to genetically obese and hyperglycemic mice (10, 11, 18). The experiments revealed that AZO02 improved hyperglycemia in a mouse model of NIDDM. BRL35135, a selective β3-adrenoceptor agonist, normalized glucose tolerance in obese Zucker rats (26). The lipolytic and insulin-releasing actions of β3-adrenoceptor agonists may be important factors in considering the etiology of their effects on the NIDDM model (26, 27). In addition, the anti-diabetic action of β3-agonists may be related to their stimulation of glucose uptake into peripheral tissues (adipose or muscle tissue). However, the precise mechanisms for how BRL35135 improves the condition of diabetic rodents have not been identified (26).

In conclusion, we demonstrated here that AZO02, a newly synthesized catecholamine derivative, is a selective β3-agonist, and it stimulates glucose uptake and lipolysis in rat adipocytes. In addition, oral administration of AZO02 to NIDDM mice improved hyperglycemia. Further pharmacological evaluation is necessary for determining the mechanism whereby AZO02 improves hyperglycemia.

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