CB1 receptor antagonist AVE1625 affects primarily metabolic parameters independently of reduced food intake in Wistar rats

Andreas W. Herling, Matthias Gossel, Guido Haschke, Siegfried Stengelin, Johanna Kuhlmann, Günter Müller, Dieter Schmoll, and Werner Kramer

Sanofi-Aventis Deutschland GmbH, Frankfurt/Main, Germany

Submitted 27 April 2007; accepted in final form 18 June 2007

Herling AW, Gossel M, Haschke G, Stengelin S, Kuhlmann J, Müller G, Schmoll D, Kramer W. CB1 receptor antagonist AVE1625 affects primarily metabolic parameters independently of reduced food intake in Wistar rats. Am J Physiol Endocrinol Metab 293: E826–E832, 2007. First published June 26, 2007; doi:10.1152/ajpendo.00264.2007.—The objective of the present study was to investigate in fed Wistar rats whether the cannabinoid-1 (CB1) receptor antagonist AVE1625 causes primary effects on metabolic blood and tissue parameters as well as metabolic rate, which are independent of reduced caloric intake. After single administration to rats postprandially, AVE1625 caused a slight dose-dependent increase in basal lipolysis. Six hours after single administration, liver glycogen content was dose-dependently reduced to ∼60% of that of untreated controls. These findings demonstrate a primary acute effect of AVE1625 on induction of 1) lipolysis from fat tissue (increased FFA) and 2) glycogenolysis from the liver (reduced hepatic glycogen). Measured by indirect calorimetry, AVE1625 caused an immediate increase in total energy expenditure, a long-lasting increase of fat oxidation, and a transient increase of glucose oxidation, which were consistent with the acute findings on metabolic blood and tissue parameters. We conclude that, in addition to the well-investigated effects of CB1 receptor antagonists to reduce caloric intake and subsequently body weight, this pharmacological approach is additionally linked to inherently increased lipid oxidation. This oxidation is driven by persistently increased lipolysis from fat tissues, independently of reduced caloric intake, and might significantly contribute to the weight-reducing effect.

cannabinoid receptors; lipolysis; glycogenolysis; energy expenditure

OBESITY COMBINED WITH ITS COMORBIDITIES has become one of the major health problems not only in industrialized but also in developing countries (16, 17, 37). Epidemiological and clinical experiences have demonstrated that dietary and behavioural treatments of obesity alone are of limited efficacy (41). Therefore, tremendous efforts in the pharmaceutical industry have been undertaken to investigate efficacious pharmacological mechanisms for the treatment of obesity. Recently, cannabinoid-1 (CB1) receptor antagonism has been intensively investigated for its potential to reduce food intake and subsequently to treat obesity (12, 31, 33). CB1 receptors are widely distributed in the central (14) and peripheral nervous systems (10) as identified for the enteric nervous system of the gut in pigs, guinea pigs, rats, and mice (23, 35), as well as in the nodose ganglion in humans, rabbits, and rats (8, 28). Furthermore, CB1 receptors are also present in several peripheral tissues (32). Recently, peripheral CB1 receptors were reported to be present on adipocytes in humans, rats, and mice (3, 9, 38), on hepatocytes (30), and on pancreatic β-cells in mice (21), and on thyroid cells in rats (36). CB1 receptors belong to the G protein-coupled receptor family and transmit their response via a Gi/0 protein with subsequent decreases in cAMP (6, 19). Accordingly, CB1 receptor antagonism is connected to increased cellular cAMP levels (27). The localization of CB1 receptors in the hypothalamus, the center of regulation of food intake and energy balance, makes it very likely that the reduced food intake after pharmacological CB1 receptor blockade is mediated by the antagonism of hypothalamic CB1 receptors (14, 32). In addition, extrahypothalamic brain areas are involved in the regulation of food intake, e.g., nucleus accumbens, where CB1 receptors were also identified (14).

Rimonabant, SR-141716, the first selective CB1 antagonist, is an effective adjunct to diet and exercise in overweight/obese patients with associated risk factors such as type 2 diabetes and dyslipidemia. In four placebo-controlled, multinational, double-blind, RIO (rimonabant in obesity) clinical studies, rimonabant has been shown to improve cardiometabolic risk factors such as waist circumference, Hb A1c, HDL, and triglyceride in overweight/obese patients (11, 18, 34, 43). About 50% of the effects of rimonabant on lipid and glycemic parameters have been shown to be independent of weight loss and may reflect direct metabolic effects of CB1 blockade in peripheral tissues (11, 43).

The objective of the present study was to investigate in fed (postprandial state) Wistar rats, whether CB1 receptor antagonism causes primary effects on metabolic blood and tissue parameters as well as metabolic rate, which are independent of reduced caloric intake by the use of AVE1625, a new CB1 receptor antagonist.

MATERIALS AND METHODS

Animals

Male Wistar rats (HsdCpb:WU) were obtained from Harlan Winkelmann (Borchen, Germany). Animals were housed in groups of three to four per cage in rooms with controlled temperature between 20 and 22°C, humidity between 55 and 65%, and on a 12:12-h light-dark cycle (lights on at 0600) with ad libitum access to standard rat chow (SSNIF, Soest, Germany) and water, if not mentioned otherwise. Groups of 7–10 rats were used for all experiments. Animal studies were performed according to the German animal protection law as well as according to international animal welfare legislation and rules.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
AVE1625 (Fig. 1) was synthesized by the chemical department of Sanofi-Aventis and was characterized as a specific and selective CB1 receptor antagonist by monitoring intracellular calcium concentrations (Fluo-4 dye) in functional assays using the FLIPR technology (Molecular Devices). Briefly, stable, transfected recombinant cells (HEK 293) expressing cannabinoid CB1 receptors from human (hCB1-R) or rat (rCB1-R) or CB2 from man (hCB2-R) as well as G protein G_{i/o} were used. Cells were incubated with AVE1625 at various concentrations followed by addition of agonist arachidonylcyclopropylamide (ACPA) in the case of CB1 (250 nM final concentration) or CP-55940 in the case of CB2 (1 μM final concentration) and measurement of triggered fluorescence signals in FLIPR. AVE1625 inhibited the agonist-stimulated calcium signal with IC_{50} values of 25 ± 4 and 10 ± 1 nmol/l for the hCB1-R and rCB1-R, respectively, and was ineffective for the hCB2-R (≥10.000 nmol/l). In the same assays, the respective values for rimonabant were 31 ± 3, 7 ± 1, and >10.000 nmol/l at the hCB1-R, rCB1-R, and hCB2-R, respectively (all values derived from 3–5 independent measurements).

Treatment and Experimental Protocols

AVE1625 was administered orally by gavage, 5 ml/kg, in a vehicle consisting of 5% solutol HS15, 0.5% hydroxymethylcellulose, and 0.2% Tween 80. The rats of the control group received only the vehicle. AVE1625 was administered to rats in a postprandial state that had free access to food and water during the preceding night (dark phase), at the beginning of the light phase (between 0700 and 0800). In separate experiments, 1) food consumption and locomotor activity up to 24 h, 2) metabolic and tissue parameters up to 6 h, and 3) energy expenditure by indirect calorimetry up to 24 h postadministration were measured after single administration of AVE1625, respectively. Although food intake is minimal during the light phase according to the physiological behavior of rats, food was withdrawn for 6 h after dosing in studies 2 and 3 to monitor primary acute effects of AVE1625 on intermediary metabolism and to exclude any acute secondary effects of AVE1625 on metabolic parameters due to reduced food consumption.

Assessment of food and water intake and locomotor activity. Rats with a body weight of 390–410 g were used for the study. Food and water intakes were assessed in rats kept single in standard cages (type III) on standard bedding, using an automated device (TSE Drinking & Feeding Monitor v. 3.26; TSE Systems, Bad Homburg, Germany). In addition, cage tops were equipped with infrared sensors (TSE InfraMot; TSE Systems) to assess general locomotor activity of the animals. Rats were habituated to the test environment in a 24-h training session prior to compound testing. Immediately after administration of AVE1625 at a dose of 30 mg/kg orally to the rats, measurements were started. Extruded food (SSNIFF R/M-H) was offered to minimize spillage, which was not further assessed. Food and water were freely and easily accessible during the study. Food and water consumption is expressed as cumulated consumption corrected to 100 g body wt. Locomotor activity was measured as arbitrary “InfraMot units.”

Metabolic blood and tissue parameters. AVE1625 was administered at doses of 3, 10, and 30 mg/kg orally to rats with a body weight of 280–320 g. Two and five hours postadministration, blood was collected from the tip of the tail for determination of glucose as well as during short-term isoflurane anesthesia retroorbitally for determination of free fatty acids (FFA), glycerol, and triglycerides. One hour later (6 h postadministration), rats were anesthetized with isoflurane, laparotomized, and killed by terminal blood collection from the aorta. Blood was used for determination of metabolic blood parameters. The liver was removed, and specimens (~1 g) were freeze-clamped in liquid nitrogen and stored at ~80°C until liver glycogen and triglyceride contents were determined.

Indirect calorimetry. The setting consisted of 16 cages, of which 15 were used for individual housing of the animals during the study, and one cage served as the reference cage for corrections of O₂ and CO₂ measurements. All rats were accustomed to the cages at least 24 h after dosing in studies 2 and 3 before testing.

![Fig. 1. Chemical structure of AVE1625.](Image 1)

![Fig. 2. Acute effects of AVE1625 after single administration of 30 mg/kg (arrow indicates time of administration in A and C) to postprandial Wistar rats on accumulated food consumption (normalized to 100 g body wt; A), adjusted per time interval (B), and locomotory activity (C). Values are means ± SE; n = 10. *P < 0.05 vs. control shown only for B.](Image 2)
before the start of the measurements (day –2) followed by a 24-h control period (day –1). AVE1625 was administered at the dose of 30 mg/kg orally. O₂ consumption (V̇O₂) and CO₂ production (V̇CO₂) were measured every 16 min/cage for 1 min (gas analyzers: Magnos 16 and Uras 14; ABB, Frankfurt/Main, Germany) and recorded using a computer. Values are expressed as the means of liters per hour of V̇CO₂ and V̇O₂, respectively. Respiratory quotient (RQ) was calculated as the quotient of V̇CO₂/V̇O₂, with the value of 1 representing 100% carbohydrate oxidation (CHox), and the value of 0.7 representing 100% fat oxidation (Fatox) (40). Total energy expenditure (TEE) was calculated according to the formula: TEE (kJ) = 16.17·V̇O₂ (l) + 5.03·V̇CO₂ (l) − 5.98·N (g), where nitrogen loss was set constantly to 0.2 g/day and expressed as TEE per metabolic body mass (kg0.75) per hour (45). Fat oxidation and carbohydrate (CH) oxidation were calculated according to Ref. 13 and modified as described in Ref. 7 by applying the following formulas: Fatox (g) = 1.72·V̇O₂ (l) − 1.72·V̇CO₂ (l) − 1.96·N (g) and CHox (g) = −2.97·V̇O₂ (l) + 4.17·V̇CO₂ (l) − 2.44·N (g) and expressed as grams per hour.

Analytic Procedures of Blood Parameters

Blood metabolic parameters were determined enzymatically using commercially available kits on a Hitachi 912 for glucose (Gluco-quant Glucose/HK kit; Roche, Germany), FFA (NEFA C kit, Wako, Germany), glycerol (Randox, Gemany), ketone bodies (autokit total ketone bodies and autokit 3-HB; Wako, Germany), and triglycerides (GPD-PAP; Roche, Germany). Standard procedures were used (4) to determine hepatic glycogen (amyloglucosidase digestion followed by glucose analysis) and hepatic triglyceride contents (lipid extraction followed by triglyceride analysis). Serum insulin concentrations were assayed with an ELISA kit (specific for rat insulin) obtained from Mercodia, Sweden.

Fig. 3. Acute effects of AVE1625 after single administration on metabolic blood parameters of fatty acid metabolism in postprandial Wistar rats: free fatty acids (FFA; A), glycerol (B), HO-butyrates (C), acetoacetate (D), serum triglycerides (E), and liver triglyceride content (F). Values are means ± SE; n = 8. *P < 0.05 vs. control.

Statistical Analysis

Data are presented as means ± SE. Depending on the homogeneity of variances (Levene test), significant differences were calculated by t-test, if appropriate, or by a one-way ANOVA followed by a post hoc Dunnett’s test or by a Kruskal-Wallis analysis. When testing for differences between the different time points, a two-way ANOVA (for repeated measures) followed by a post hoc Dunnett’s test was used. For all statistical calculations the software package Eversat v. 5 (Sanofi-Synthelabo based on SAS 8) was used. P < 0.05 was considered to be statistically significant.

RESULTS

At the beginning of the light phase, acute administration of AVE1625 at the dose of 30 mg/kg orally to rats that had free access to food during the preceding night (postprandial state) caused a pronounced reduction of food intake during the subsequent 10 –12 h (Fig. 2, A and B) without differences in their locomotor activity relative to that of the control group (Fig. 2C). During the 12-h light phase, food consumption was reduced by more than 50% relative to that of the control group, whereas reduction of food uptake in the first half of the dark phase was still significant but less pronounced (Fig. 2B). These results demonstrated that the pharmacological effect of the CB1 receptor antagonist AVE1625 to reduce food consumption might have been underestimated when administered to rats in the postprandial state during the light phase. However, this study design was chosen to investigate whether a CB1 receptor
antagonist has additional direct pharmacological effects on metabolic parameters.

Therefore, we investigated the primary acute effect of AVE1625 on metabolic parameters in fed rats in the postprandial state at the beginning of the light phase, when food intake is physiologically very low in rats. Furthermore, to exclude any secondary effects of reduced food uptake, which might occur even at the beginning of the light phase, on metabolic blood and tissue parameters, food was removed from all study groups. Administration of AVE1625 to these rats caused an increase in FFA and glycerol, indicating increased lipolysis from fat tissue (Fig. 3, A and B). In parallel to the increase in FFA, ketone bodies were also increased, as measured at the end of the study period after only 6 h (Fig. 3, C and D). Surprisingly, despite elevated FFA levels, serum triglyceride levels did not increase during the 6-h study period (Fig. 3E), and hepatic triglyceride content was not affected after 6 h (Fig. 3F). After 2 h, blood glucose was slightly elevated but was normalized 5 h after administration (Fig. 4A), despite reductions of liver glycogen down to ~60% of that of the control group at the highest tested dose 6 h after administration (Fig. 4C). In addition, serum insulin did not change (Fig. 4B). In summary, these findings demonstrated an acute primary effect of AVE1625 on induction of 1) lipolysis from fat tissue (increased FFA) and 2) glycogenolysis from the liver (reduced hepatic glycogen), without any changes in serum triglyceride levels or meaningful increases of blood glucose; these findings were clearly not secondary due to reduced food intake, as food was removed during the 6-h study period.

Next, we investigated in a single-dose study the acute effect on TEE by measuring $\dot{V}O_2$ and $\dot{V}CO_2$ in the setting of indirect calorimetry in fed rats that also had no access to food for 6 h postadministration of vehicle or AVE1625. Differences in the energy metabolism during this phase were not due to the inhibition of food intake by AVE1625. Administration of AVE1625 at the dose of 30 mg/kg orally immediately resulted in a pronounced increase in $\dot{V}CO_2$ and $\dot{V}O_2$, indicating increased oxidation of energetic substrates and increased TEE (Fig. 5, A–C). Calculation of the RQ demonstrated a pronounced and long-lasting shift to increased lipid oxidation (Fig. 5D). Detailed calculation of fat and glucose oxidation rates revealed a pronounced and long-lasting increase in fat oxidation (Fig. 5E), whereas glucose oxidation initially demonstrated the tendency to be elevated over 3–4 h but thereafter fell below that of the control group (Fig. 5F).

**DISCUSSION**

Rats are nocturnal animals and therefore take up about two-thirds of their daily food during the dark phase. The study window of pharmacological inhibition of food consumption is much lower in rats when they are treated with a test compound at the beginning of the light phase, which means a postprandial state of the animals. Even under this unfavorable study condition, the pharmacological effect to reduce food intake could be clearly demonstrated for the CB1 receptor antagonist AVE1625. Furthermore, locomotor activity was not influenced by the compound, which evidently demonstrated that the reduced food intake was not caused by other confounding factors, e.g., general malaise or central depression. This study design was selected to investigate the primary pharmacological effect of CB1 receptor antagonism on blood and tissue parameters of intermediary metabolism. Thus, in the two other studies, any secondary effects of reduced food intake on metabolic blood and tissue parameters as well as during the indirect calorimetric study were excluded by withdrawing food for 6 h after administration to postprandial rats, for both control and treated groups.

According to the normal physiological light-dark behavior of rats, energy expenditure followed this circadian rhythm: $\dot{V}O_2$ and $\dot{V}CO_2$ were elevated during the dark phase relative to those of the light phase. AVE1625 administered at the beginning of the light phase to rats in a postprandial state caused an immediate increase in TEE despite no food intake. Thus, AVE1625 primarily affects metabolic rate, without increasing locomotor activity of the rats. The increased energy expenditure was based predominantly on a pronounced long-lasting increase of fat oxidation and on a transient minor increase of glucose oxidation. These findings confirm previously described results on $\dot{V}O_2$ with rimonabant, the best-characterized CB1 antagonist.
receptor antagonist so far, monitored for a short period of only 3 h in Lep<sup>ob</sup>/Lep<sup>ob</sup> mice (24).

In single-dose studies, the results on metabolic blood and tissue parameters were consistent with the finding of increased energy expenditure. AVE1625 induced 1) an immediate adipocytic lipolysis resulting in slightly increased levels of FFA, glycerol, and ketone bodies within a few hours after administration without changes in serum insulin and 2) an acute glycogenolysis from the liver measured 6 h postadministration. Blood glucose was only slightly elevated at the highest tested dose 2 h after administration and was normalized 3 h later. Consistently glucose oxidation was only initially and slightly increased as measured by indirect calorimetry.

Increased levels of FFA are generally interpreted as deleterious, because they are associated with an increased supply of fatty acids to nonadipose tissue such as liver and muscle as well as with increased levels of serum triglycerides. Presently, ectopic triglyceride accumulation is thought to be involved in hepatic (1, 25, 39) and muscle insulin resistance (2, 5, 15, 20, 22, 44). However, despite slightly elevated levels of FFA, serum triglycerides were not elevated in our studies. In addition, liver triglyceride content, as measured by biochemical analysis, was not increased.

The acute and direct effects of AVE1625 to induce lipolysis and glycogenolysis simultaneously in the presence of concomitantly increased energy expenditure were not mediated by reduced caloric intake, because the rats were in a postprandial state and food was not available during the study period of 6 h. Therefore, the question arises from these findings: how are these effects of AVE1625 on metabolic parameters mediated? One explanation could be direct antagonism of peripheral CB1 receptors on adipocytes and hepatocytes, because the receptor expression on these two cell types has been reported (3, 30). Both lipolysis and glycogenolysis are related to increased cAMP levels, which would be consistent with the knowledge that CB1 receptors are coupled to Gi/0 protein (6, 19), and therefore receptor antagonism increased intracellular cAMP levels (27). A second explanation could be that these effects are caused by the well-investigated neuromodulatory function of CB1 receptors (26) resulting in disinhibition of transmitter release at peripheral sympathetic nerve endings in adipose tissue and the liver due to presynaptic CB1 antagonism. A further explanation could be that these fine-tuned effects on lipolysis and glycogenolysis were caused by coordinated central mediation to peripheral metabolic tissues, e.g., via increased sympathetic outflow from the hypothalamus (29), be-

Fig. 5. Acute effects of AVE1625 (30 mg/kg) after single administration (arrow indicates administration) on CO2 production (V<sub>CO2</sub>; A), O2 consumption (V<sub>O2</sub>; B), total energy expenditure (TEE; C), respiratory quotient (RQ; D), and fat (E) and carbohydrate (CH) oxidation (F) in postprandial Wistar rats. Animals had free access to food except for the 1st 6 h after administration of AVE1625 or vehicle. Values are means ± SE; n = 7–8.
cause it has been reported that CB1 receptor antagonism increases norepinephrine outflow in the rat anterior hypothalamus (42). We conclude from these studies that AVE1625 primarily affects peripheral intermediary metabolism by inducing hepatic glycogenolysis and glucose oxidation transiently as well as by inducing longer-lasting lipolysis from fat tissue and lipid oxidation independently of its effect to reduce food intake. Further investigations are needed to clarify whether these pharmacological activities are centrally (e.g., hypothalamically) or peripherally mediated. In addition to the well-investigated effect of CB1 receptor antagonists to reduce body weight due to reduced caloric intake, we identified increased lipid oxidation driven by persistently increased lipolysis in fat tissue as an additional pharmacological end point of CB1 receptor antagonism. This increased lipolysis from fat tissue, combined with increased fat oxidation, might contribute to the weight-reducing effect of CB1 receptor antagonists such as AVE1625 or rimonabant, and their beneficial effects on metabolic parameters.

DISCLOSURES

The authors are employed by and hold stock in Sanofi-Aventis Deutschland GmbH.

REFERENCES


