A₁ adenosine receptor partial agonist lowers plasma FFA and improves insulin resistance induced by high-fat diet in rodents

Arvinder K. Dhalla,¹ Mei Yee Wong,¹ Peter J. Voshol,² Luiz Belardinelli,¹ and Gerald M. Reaven³

¹Department of Pharmacological Sciences, CV Therapeutics, Palo Alto, California; ²Department of Endocrinology and Metabolic Diseases, Leiden University Medical Center, Leiden, The Netherlands; and ³Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, California

Submitted 24 October 2006; accepted in final form 10 January 2007

Dhalla AK, Wong MY, Voshol PJ, Belardinelli L, Reaven GM. A₁ adenosine receptor partial agonist lowers plasma FFA and improves insulin resistance induced by high-fat diet in rodents. Am J Physiol Endocrinol Metab 292: E1358–E1363, 2007. First published January 16, 2007; doi:10.1152/ajpendo.00573.2006.—There is substantial evidence in the literature that elevated plasma free fatty acids (FFA) play a role in the pathogenesis of type 2 diabetes. CVT-3619 is a selective partial A₁ adenosine receptor agonist that inhibits lipolysis and lowers circulating FFA. The present study was undertaken to determine the effect of CVT-3619 on insulin resistance induced by high-fat (HF) diet in rodents. HF diet feeding to rats for 2 wk caused a significant increase in insulin, FFA, and triglyceride (TG) concentrations compared with rats fed chow. CVT-3619 (1 mg/kg) caused a time-dependent decrease in fasting insulin, FFA, and TG concentrations. Acute administration of CVT-3619 significantly lowered the insulin response, whereas glucose response was not different with an oral glucose tolerance test. Treatment with CVT-3619 for 2 wk resulted in significant lowering of FFA, TG, and insulin concentrations in rats on HF diet. To determine the effect of CVT-3619 on insulin sensitivity, hyperinsulinemic euglycemic clamp studies were performed in C57BL/6J mice fed HF diet for 12 wk. Glucose infusion rate was decreased significantly in HF mice compared with chow-fed mice. CVT-3619 treatment 15 min prior to the clamp study significantly (P < 0.01) increased glucose infusion rate to values similar to that for chow-fed mice. In conclusion, CVT-3619 treatment lowers FFA and TG concentrations and improves insulin sensitivity in rodent models of insulin resistance.

CVT-3619; antilipolytic; free fatty acids; triglycerides; oral glucose tolerance test

INCREASES IN PLASMA FFA CONCENTRATIONS are characteristic of insulin-resistant individuals (28), and this generalization applies to both nondiabetic persons as well as patients with type 2 diabetes (6, 14, 28, 35). Furthermore, there is evidence (4, 13, 24, 28) that increases in plasma FFA concentrations play a role in the pathogenesis of both type 2 diabetes and cardiovascular disease. There are at least three explanations for the adverse clinical consequences of elevated circulating FFA concentrations. First, increased plasma FFA concentrations decrease insulin-mediated glucose disposal by muscle (12, 32). Second, an increase in FFA flux to the liver stimulates hepatic triglyceride (TG) synthesis and secretion, and the more hyperinsulinemic the individual, the greater the hypertriglyceridemic response to the increase in plasma FFA concentration (18, 22, 30). Furthermore, the ensuing hypertriglyceridemia is associated with a decrease in high-density lipoprotein cholesterol concentration, a shift to smaller and denser low-density lipoprotein particles, and an exaggerated postprandial accumulation of TG-rich remnant lipoproteins, leading to a highly atherogenic lipoprotein profile (29). Finally, there is evidence in both animals and humans that glucose-stimulated insulin secretion is reduced in response to chronic elevations of plasma FFA concentrations (5, 20, 33).

Given this background, it would seem reasonable to suggest that pharmacological lowering of elevated FFA concentrations would have considerable clinical benefit. In this regard, acipimox, an analog of nicotinic acid that inhibits lipolysis and can acutely lower circulating FFA concentrations, improves insulin sensitivity (2, 34). However, acipimox had no effect on glucose and insulin concentrations, whereas plasma FFA concentrations were increased after a 3-mo treatment that was suggested to be due to rebound lipolysis (36). Similarly, nicotinic acid has also been associated with the induction of an insulin-resistant state, presumably due to a rebound increase in plasma FFA concentrations (20, 25). Thus, finding a suitable pharmacological agent to lower FFA remains a challenge.

Activation of A₁ adenosine receptors has been shown to inhibit lipolysis and lower circulating FFA concentrations via inhibition of adenylyl cyclase activity and reduction in cAMP (7). Transgenic mice overexpressing A₁ receptors in adipose tissue had lower plasma FFA concentrations and improved insulin sensitivity compared with wild type on a high-fat (HF) diet (9). Thus, adenosine and some of its analogs that bind to the A₁ receptor on adipocytes (1, 11) offer an alternative therapeutic approach to lower plasma FFA concentrations (8). Evidence that adenosine analogs may have clinical utility is supported by the results showing that phenylisopropyladenosine administration causes a fall in FFA concentrations, hepatic very-low density lipoprotein-TG secretion rate, and TG concentrations in normal and hypertriglyceridemic rats (17). However, the fact that these compounds can have undesirable cardiovascular effects has decreased interest in their potential therapeutic utility (3). On the other hand, recent evidence (8, 11) has suggested that it is possible for A₁ agonists to have significant antilipolytic effects at doses that have no or minimal cardiac effects. Furthermore, partial agonists of the A₁ receptor elicit submaximal responses at high concentrations and cause less receptor desensitization than full agonists (38, 39). Therefore, partial agonists may be particularly attractive as potential therapeutic agents. CVT-3619 (a novel derivative of adeno-
Glucose at PBS solution was started at hyperinsulinemic clamp started by continuous infusion of insulin. Thereafter, a bolus of insulin was given and a turnover studies were performed as described previously (10, 15). CVT-3619 was given via an intraperitoneal injection 15 min before sure insulin sensitivity in the absence and presence of CVT-3619. A hyperinsulinemic euglycemic clamp analysis was performed to mea-

lard (8) for 12 wk to induce insulin resistance. At the end of 12 wk, a group were given a diet (TD88137; Harlan-Teklad, Madison, WI) containing 42% fat, 43% carbohydrate, and 15% protein. The antilipolytic effects of CVT-3619 (see chemical name below) were studied in awake rats. On the day of the experiment, animals were put in metabolic cages and left undisturbed to acclimate to the environment for 1–2 h. An infusion set (21G × 3/4 in., 0.8 × 19 mm UTW, 3 1/2 inches, 9-cm tubing, volume 0.15 ml) was connected to the arterial catheter for blood sampling. A 1% sodium citrate saline solution was used to flush the lines. A pretreatment blood sample was obtained from each animal to determine baseline values for glucose, insulin, FFA, and TG. Blood samples were collected into plasma and serum separator tubes (Becton-Dickinson, Franklin Lakes, NJ) at predetermined time points. Oral glucose tolerance test (OGTT) was performed by giving 2 gm/kg of glucose load. Because the half-life of CVT-3619 is relatively short (~2 h) due to high clearance (40–60 ml·min⁻¹·kg⁻¹), CVT-3619 was given via oral gavage 15 min prior to the glucose load. For chronic experiments CVT-3619 was administered twice a day via subcutaneous injection at a dose of 5 mg/kg for 2 wk. An OGTT was performed at the end of 2 wk (~2 h) after the last dose of CVT-3619. Doses of CVT-3619 used in the study were determined from previous studies (8) and had no cardiovascular effects.

Mouse studies. C57BL/J6 mice were maintained on normal chow or a HF diet (bovine lard; 23% wt/wt, 44% energy provided by the lard) for 12 wk to induce insulin resistance. At the end of 12 wk, a hyperinsulinemic euglycemic clamp analysis was performed to measure insulin sensitivity in the absence and presence of CVT-3619. CVT-3619 was given via an intraperitoneal injection 15 min before the clamp protocol was started. After an overnight fast, glucose turnover studies were performed as described previously (10, 15). Briefly, animals were anesthetized; an infusion needle was placed in one of the tail veins. Thereafter, a bolus of insulin was given and a hyperinsulinemic clamp started by continuous infusion of insulin. Blood samples were taken every 10 min (tail bleeding) to monitor plasma glucose levels. A variable infusion of 12.5% d-glucose (in PBS) solution was started at time 0 and adjusted to maintain blood glucose at ~6.0 mM. When steady-state glucose levels were reached (~1 h after start of the insulin infusion) a final blood sample was taken for measurement of plasma insulin, and the hyperinsulinemic euglycemic clamp was terminated. There were no significant differences in blood glucose or plasma insulin concentrations between the three groups of mice during the clamp analysis.

Chemicals. CVT-3619 (2-[6-[(1R,2R)-2-hydroxycyclopentyl]amino]purin-9-yl)(4S,5S,2R,3R)-5-[(2-fluorophenylthio)methyl]oxolane-3, 4-diol) was synthesized by the Department of Medicinal and Bio-Organic Chemistry of CV Therapeutics, and PEG 400 was purchased from VWR (by EMD Chemicals). CVT-3619 was dissolved in PEG 400 and then diluted with distilled water to make a 20% PEG drug solution. FFA and TG concentrations were measured using commercial kits from Wako Chemicals (Richmond, VA). Glucose and insulin concentrations were measured using commercial kits from Thermo Electron (Waltham, MA) and Crystal Chem (Downers Grove, IL), respectively.

Data analysis. All data are reported as means ± SE. Statistical analysis of data from experiments with two treatment groups was performed using the unpaired Student’s t-test. One-way analysis of variance followed by Newman-Keuls post hoc analysis was used for multiple comparisons. Results of the OGTT were analyzed by calculating area under the curve with the use of prism graphpad software. Differences between/among treatment groups were considered to be significant when the probability of their occurrence by chance alone was <0.05.

RESULTS

Effect of HF diet. Table 1 presents the weight and metabolic characteristics after 2 wk in which rats were fed either conventional chow or the HF diet. It can be seen that there were no significant differences in either the body weight or the plasma glucose concentrations of the two groups (Table 1). However, insulin, FFA, and TG concentrations were all significantly higher in rats fed the HF diet compared with the rats fed chow.

Acute studies in rats. The acute effects of an oral administration of CVT-3619 (1 mg/kg) on plasma insulin, FFA, and TG concentrations in rats fed either chow or the HF diet are shown in Fig. 1. Consistent with the results in Table 1, baseline concentrations of these three variables were higher in the HF-fed rats. Although insulin, FFA, and TG concentrations fell promptly in response to CVT-3619 in both groups, the results in Table 2 show that the magnitude of the response was greater for all three variables in the HF group. Consequently, insulin, FFA, and TG concentrations were essentially identical in the two groups from the 60-min time point to the end of the experiment.

Figure 2 depicts the glucose and insulin responses to an oral glucose load in three groups of rats fed either chow (1 group) or the HF diet (2 groups) for 2 wk. Chow-fed rats were gavaged with vehicle, whereas one group of the rats fed the HF diet received vehicle, and the other group was given CVT-3619 15 min prior to giving the glucose load. Glucose concentrations are shown in Fig 2A, and there were no differences between the total glucose response areas of the three experimental groups. Postglucose challenge insulin concentrations are shown in Fig. 2B and indicate that the total insulin response area in the saline-treated HF diet group was significantly greater than that of the chow-fed vehicle-treated rats (P < 0.01). The total insulin response of HF-fed rats treated with CVT-3619 was

<table>
<thead>
<tr>
<th>Table 1. Baseline characteristics of Sprague-Dawley rats fed normal chow (Chow) and HF diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
</tr>
<tr>
<td>FFA, mM</td>
</tr>
<tr>
<td>TG, mg/dl</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. HF, high-fat diet; FFA, free fatty acids; TG, triglycerides. Rats were fasted for 4 h before taking blood samples for glucose, insulin, FFA, and TG analysis.
significantly (P < 0.01) lower than that of the HF-fed rats treated with vehicle and was no different than the insulin response of chow-fed rats given vehicle (P > 0.05). There was a small decrease in insulin response in chow-fed rats treated with CVT-3619 that was not significantly different from chow-fed vehicle-treated group (data not shown).

**Mouse studies.** By inference, the results described above are consistent with evidence that insulin resistance develops in rats fed a HF diet and that administration of CVT-3619 attenuates the diet-induced impairment in insulin action. Hyperinsulinemic euglycemic clamp studies were performed to test this hypothesis. The results in Fig. 4 demonstrate that insulin-mediated glucose disposal was decreased significantly in mice fed the HF diet for 12 wk compared with chow-fed mice. However, the intraperitoneal injection of two different doses of CVT-3619 15 min prior to beginning the clamp study enhanced insulin sensitivity, with CVT-3619 that was not significantly different from chow-fed vehicle-treated group (data not shown).

**Chronic studies in rats.** The effect of chronic treatment with CVT-3619 compared with vehicle placebo on fasting glucose, insulin, FFA, and TG concentrations is shown in Fig. 3. Although there were no significant differences in glucose concentrations, rats fed a HF diet had significantly lower insulin, FFA, and TG concentrations when they received daily subcutaneous injections of CVT-3619 (5 mg/kg) for 2 wk. Plasma glucose and insulin responses to an oral glucose challenge in HF-fed rats following a 2-wk period in which they received either subcutaneous injections of vehicle or CVT-3619 (5 mg/kg) twice were not different from vehicle-treated rats (data not shown).

Table 2. Decrease in insulin FFA, and TG, concentrations from 0 to 30 min following CVT-3619 treatment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, ng/ml</td>
<td>Chow</td>
<td>0.8±0.32</td>
</tr>
<tr>
<td>FFA, mM</td>
<td>HF</td>
<td>2.37±0.85</td>
</tr>
<tr>
<td>TG, mg/dl</td>
<td>Chow</td>
<td>0.24±0.07</td>
</tr>
<tr>
<td></td>
<td>HF</td>
<td>17±3</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. Rats were fasted for 4 h before taking the baseline sample. CVT-3619 was given by an oral gavage at a dose of 1 mg/kg.
and the values of insulin-mediated glucose disposal in the CVT-3619-treated mice were significantly greater \((P < 0.01)\) than in saline-injected mice fed a HF diet and no different than in chow-fed mice. It should be noted that both doses (2.5 and 5 mg/kg) of CVT-3619 increased glucose infusion rate to a similar extent.

**DISCUSSION**

Previously, we (8, 11) have shown that CVT-3619 is a selective and partial \(A_1\) adenosine receptor agonist with anti-lipolytic activity. CVT-3619 inhibited cAMP accumulation and FFA release from rat adipocytes (11) and lowered FFA and TG in a dose-dependent manner in rats, and the FFA-lowering effect was not associated with a rebound or cardiovascular effects (8). Furthermore, CVT-3619 potentiated the reduction in plasma FFA concentrations caused by insulin (8).

The objective of the present study was to determine the potential clinical utility of the FFA-lowering effects of CVT-3619 by studying its effects in insulin-resistant animal models. The basic premise underlying this approach is that circulating FFA concentrations are elevated in insulin-resistant individuals (6, 14, 28, 35) and that lowering these values toward normal will both enhance insulin sensitivity and decrease TG concentrations. The results presented support this general formulation and suggest that CVT-3619 may provide a useful pharmacological approach with which to prevent some of the abnormalities and adverse clinical syndromes that occur more frequently in insulin-resistant individuals (26, 27).

To begin with, there is evidence (21, 31) that insulin resistance develops when rats or mice are fed a HF diet. Results of the current study are consistent with these previous findings and provide direct evidence that 1) insulin-mediated glucose disposal decreases in mice fed a HF diet (Fig. 4); 2) feeding the HF diet to rats leads to elevated insulin, FFA, and TG concentrations (Table 1); and 3) a hyperinsulinemic response to an oral glucose challenge is seen in HF-fed rats (Fig. 2). It should be emphasized that these changes were seen despite the fact that the body weights of chow-fed and HF-fed rats were not different. Thus, this animal model of insulin resistance is not the consequence of diet-induced obesity.

Of greater relevance to the goal of this study is that administration of CVT-3619 attenuated the adverse metabolic changes in rats rendered insulin resistant by consuming a HF diet. CVT-3619 can acutely enhance insulin sensitivity during
the clamp study in insulin-resistant HF-diet fed mice (Fig. 4), lower insulin, FFA, and TG concentrations in rats fed a HF diet (Fig. 1), and decrease insulin responses to an oral glucose load in HF diet-induced insulin-resistant rats (Fig. 2). Furthermore, chronic administration of CVT-3619 to HF-fed rats decreased fasting insulin, FFA, and TG concentrations (Fig. 2).

In conclusion, CVT-3619 administration is able to improve insulin sensitivity and lower TG concentrations in nonobese rodent models of insulin resistance. It is assumed that these beneficial metabolic changes are secondary to the antilipolytic action of this partial adenosine receptor agonist (11), leading to lower circulating FFA concentrations and the improvement in insulin action and decrease in TG concentrations. Thus, two of the potential adverse consequences of elevated FFA concentrations (30, 32) were significantly reduced in these rodent models of insulin resistance. These studies were not planned to determine whether administration of CVT-3619 might also attenuate the downregulation of glucose-stimulated insulin secretion that occurs with chronic elevations of FFA concentrations (5, 33), and this possibility remains to be evaluated. In any event, if these findings can be reproduced in humans, while at the same time avoiding the rebound increase in FFA concentrations and adverse cardiovascular effects associated with other antilipolytic agents, CVT-3619, or similar partial A1 agonists, may offer a new and important therapeutic approach to clinical syndromes characterized by insulin resistance.

CVT-3619, being a partial agonist for the A1 receptors, may have the following advantages over full agonists: 1) increased organ/tissue selectivity, resulting in reduced unwanted effects; 2) less pronounced receptor desensitization, which is important for chronic treatment; 3) broader therapeutic dose range; and 4) partial blockade of the untoward effects of endogenous full agonist under certain pathological conditions. Consequently, it is possible to achieve a greater functional selectivity using partial agonists than full agonists; e.g., CVT-3619 does not significantly affect heart rate and blood pressure at doses that have significant antilipolytic activity (8). Furthermore, partial agonists of G protein-coupled receptors have been suggested to cause less pronounced receptor desensitization (19, 37) than full agonists with prolonged and continuous exposure (16, 23). In that context, we have shown that the antilipolytic effects (the magnitude and the duration the FFA-lowering effect) of CVT-3619 are well-maintained over three consecutive acute administrations (8); however, it remains to be determined whether the antilipolytic effect of CVT-3619 is sustained over long-term use. Since partial agonists have a broader therapeutic dose range, i.e., the difference between the effective doses needed to lower FFA concentrations and to depress cardiac function is greater for partial than for full agonists, making them potentially much safer drugs, and the current data also indicate that they retain significant metabolic efficacy.

REFERENCES
24. Pirro M, Mauriege P, Tchernev A, Cantin B, Dagenais GR, Despres JP, Lamarche B. Plasma free fatty acid levels and the risk of ischemic


