Partial A1 Adenosine Receptor Agonist Regulates Cardiac Substrate Utilization in Insulin-Resistant Rats in Vivo

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**ABSTRACT**

Reducing the availability and uptake of fatty acids is a plausible pharmaceutical target to ameliorate glucose intolerance and insulin resistance. CVT-3619 [2-[(1R,2R)-2-hydroxycyclopentyl]amino]purin-9-yl[(4S,5S,2R,3R)-5-[(2-fluorophenylthio)methyl]oxolane-3,4-diol] is a partial A1 adenosine receptor agonist with antilipolytic properties. Aims of the present study were to examine the acute effects of CVT-3619 on whole-body and cardiac glucose and fatty acid kinetics in vivo in normal and diet-induced insulin-resistant rats. Male Sprague-Dawley rats were fed either a chow (CH) or high-fat (HF) diet for 4 weeks. Catheters were then chronically implanted in the carotid artery and jugular vein for sampling and infusions, respectively. After 5 days of recovery, fasted animals (10 h) received either saline or CVT-3619 (0.4 mg/kg bolus + 1 mg/kg/h). Indices of glucose and fatty acid utilization were obtained by the administration of 2-deoxy[14C]glucose and [9,10-3H]2-bromopalmitate. HF feeding resulted in elevated, fasting insulin and free fatty acid (FFA) levels compared with CH. CVT-3619 caused a 64 and 86% reduction of FFA and insulin in HF (p < 0.05) but less (N.S.) in CH diet-fed animals. In HF diet-fed rats, CVT-3619 increased whole-body glucose clearance with no change in fatty acid kinetics. Likewise, analysis of cardiac tissue metabolism showed that CVT-3619 caused an increased glucose but not fatty acid clearance in HF-fed animals. Results show that the acute administration of CVT-3619 lowers circulating fatty acid levels, leading to improved whole-body and cardiac glucose clearance in a model of diet-induced insulin resistance. As such, CVT-3619 may be a treatment option for the restoration of substrate balance in the insulin-resistant heart.

Insulin resistance is characterized by elevated circulating free fatty acids (FFAs) as well as increases in the utilization and efficiency of lipids as a substrate (Hegarty et al., 2002). These changes in metabolism impair insulin action, alter metabolic gene expression, and promote the accumulation of intracellular triglycerides (Kelley and Goodpaster, 2001; Finck et al., 2002). The myocardium is particularly affected by this shift in metabolism, with approximately 60% of individuals with type 2 diabetes developing some form of cardiac dysfunction (Poirier et al., 2001; Aasum et al., 2003). Forced to alter both its structure and biochemistry to compensate for these changes, the heart becomes increasingly susceptible to injury when exposed to ischemia (Stamler et al., 1993; Diamant et al., 2003). Given this, reducing FFA availability and tissue utilization is a plausible pharmaceutical approach for the treatment of insulin resistance.

A number of compounds have been developed for this purpose, including A1 adenosine receptor agonists (van Schaick et al., 1997; Dhalla et al., 2003; Lam and Lopaschuk, 2007). Activation of A1 adenosine receptors inhibits lipolysis and lowers plasma FFA concentrations by inhibiting adenylyl cyclase and downstream cAMP formation (Dhalla et al., 2003). It is unfortunate that the majority of full A1 agonists also have significant cardiovascular effects. For this reason, selective but partial A1 adenosine receptor agonists have been developed (Dhalla et al., 2003, 2007a). CVT-3619 is a partial A1 adenosine receptor agonist that has antilipolytic effects at concentrations that are not accompanied by significant cardiovascular effects (Fatholahi et al., 2006; Dhalla et al., 2007a). Desensitization of adenosine receptors to chronic drug exposure is also minimal with partial agonists (Wu et al., 2007).
al., 2001). For this reason, CVT-3619 is an attractive molecule for the treatment of hyperlipidemia, insulin resistance, and related metabolic disorders. Recent studies show that CVT-3619 can lower circulating FFA, improve insulin sensitivity, and potentiate insulin action (Dhalla et al., 2007b). Employing a hyperinsulinemic-euglycemic clamp in mice, Dhalla et al. (2007b) demonstrated that an acute dose of CVT-3619 restored insulin sensitivity to levels comparable with controls. Similar findings have also been found with chronic dosing. Two weeks of CVT-3619 administration to insulin-resistant rats lowers plasma insulin, FFA, and tri-glyceride concentrations (Dhalla et al., 2007b).

In the present study, isotopic analogs were employed to examine the specific actions of CVT-3619 on substrate kinetics in control and insulin-resistant rats. In vivo indices of glucose and fatty acid utilization were simultaneously assessed using 2-deoxy[14C]glucose and [9,10-3H]-(R)-2-bromopalmitate. Results show CVT-3619 to cause an improvement in both whole-body and cardiac glucose disposal, suggesting that the A₁ adenosine receptor agonist may be of utility for the restoration of substrate balance in insulin resistance.

Materials and Methods

Animals. All procedures were approved by the University of California Animal Care and Use Subcommittee and followed Canadian Council Animal Care guidelines for the care and use of laboratory animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed individually and maintained at 23±C on a 6:00 AM to 6:00 PM light cycle. Rats were randomly divided into two diet groups and fed ad libitum standard chow (CH) (Labdiet; Purina, St. Louis, MO) or a high-fat, high-sucrose diet (Testdiet 58R3; Purina) for 4 weeks. Energy density (percentage of kilocalories per gram) for chow and high-fat diets were 23% protein, 21% fat, and 55% carbohydrate and 15% protein, 59% fat, and 26% carbohydrate, respectively.

Surgical Procedures. Animal surgeries were conducted after the 3 weeks of dietary manipulation, 5 days before the experimental protocol. Surgical procedures were performed as described previously for arterial and venous catheterizations (Petersen et al., 2003). In brief, animals were anesthetized with a 50:5:1 (v/v) mixture of ketamine, rompun, and acepromazine, and the left common carotid artery and right jugular vein were catheterized with PE50 tubing. Catheters were exteriorized and secured at the back of the neck, filled with heparinized saline (150 U/ml), and sealed with a stainless steel plug. Immediately post surgery, each animal received 75 mg/kg s.c. ampicillin to prevent infection. After surgery, animal weights and food intake were monitored daily, and only the animals in which pressure weight was restored were used for experiments.

Isotopic Analogs. [9,10-3H]-(R)-2-bromopalmitate ([3H]BROMO) (American Radiolabeled Chemicals, St. Louis, MO) was synthesized by the Department of Medicinal and Bio-Organic Chemistry of CV Therapeutics. CVT-3619 was dissolved in PEG 400 (Sigma-Aldrich) and diluted in distilled water.

Partial A₁ Adenosine Receptor Agonist. CVT-3619 was synthesized by the Department of Medicinal and Bio-Organic Chemistry of CV Therapeutics. CVT-3619 was dissolved in PEG 400 (Sigma-Aldrich) and diluted in distilled water.

Experimental Procedures. On the day of the study, rats were fasted for 10 h and given free access to water. Approximately 1 h before the experiment, catheters were flushed with heparinized saline (10 U/ml) and connected to PE50 and silastic tubing for infusions and sampling. Rats were then placed back in the cage until the commencement of the experimental protocol. Throughout the experimental protocol, rats were conscious and unrestrained. At time −100 min, baseline blood samples (B) were obtained (100 µl) for the measurement of plasma glucose, insulin, and nonesterified fatty acids (NEFAs). After baseline sampling, animals received either an infusion of saline or a primed constant infusion of CVT-3619 for 100 min (0.4 mg/kg bolus + 1 mg/kg/h). This period allowed ample time for inhibition of lipolysis by CVT-3619 to occur (Pathalobi et al., 2006). To prevent declines in hemocrit, the erythrocytes taken before isotopic analog infusion were washed in saline and reinfused shortly after each sample was taken. After infusions, a plasma sample (100 µl) was obtained (time 0 min).

Plasma Analysis. Plasma NEFAs were measured spectrophotometrically by an enzymatic colorimetric assay (Wako NEFA C kit; Wako Biochemistry, Richmond, VA). Plasma glucose concentrations were measured by the glucose oxidase method using an automated glucose analyzer (Beckman Coulter, Fullerton, CA). Immunoreactive insulin was measured in samples obtained at baseline (time 0) and at 30 min using a double-antibody method (Millipore Corporation, Billerica, MA). [3H]BROMO and [2-14C]DG were administered as a bolus into the jugular vein. Additional plasma samples (200 µl) were obtained from the carotid artery at 7, 10, 15, 20, and 30 min for the measurement of each isotopic analog, plasma glucose, and NEFA. After the last blood sample, rats were anesthetized with pentobarbital sodium, and the heart was rapidly excised, rinsed in saline to remove excess blood, freeze-clamped in liquid nitrogen, and kept frozen at −80°C until further analysis.

Tissue Analysis. Hearts were homogenized in 2 ml of 0.5% perchloric acid and centrifuged for 20 min. Supernatants (1.5 ml) were then neutralized using 5 M KOH, and radioactivity in a 250-µl sample was determined by liquid scintillation counting (Packard Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA) using a Packard Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer Life and Analytical Sciences).

Calculation. In vivo metabolism was determined by the tissue-trapping principle, using radioactive nonmetabolizable analogs of glucose and FA. Long-chain FA clearance (Rg) and metabolic index (Rm) were calculated from the accumulation of [3H]BROMO in tissues (m) and the integral of the plasma (p) disappearance after the bolus. Likewise, glucose clearance (Kg) and metabolic index (Rg) were calculated from the accumulation of [2-14C]DG in tissues (m) and the integral of the plasma (p) disappearance. The dose of tracer is represented by D. These measurements have been described previously (Dhalla et al., 2001). Calculations employed were as follows, where D(t) represents the time between 7 and 30 min.

$$K_g = \left[\frac{[3H]BROMO]_p}{[3H]BROMO]_m}\right] \int_0^t D(t)dt$$

(1)

$$K_g = [2-14C]DG]_p \int_0^t [2-14C]DG]_m D(t)dt$$

(2)

$$R_g = K_g \times [LCFA]_p$$

(3)

$$R_g = K_g \times [Glucose]_p$$

(4)
tracer administered (D) and the disappearance of tracer in the plasma (p). Rates of whole-body utilization (U) are calculated by MCR multiplied by the tracee. All whole-body measures were normalized to body weight of the animal and are expressed per 100 g. Equations are described as follows:

$$MCR_p = \frac{D}{\alpha_p} \int [1^1H]BROMO_p \, dt$$  \hspace{1cm} (5)

$$MCR_f = \frac{D}{\alpha_f} \int [2-14C]DG_p \, dt$$  \hspace{1cm} (6)

$$U_p = MCR_p \times [LCFA]_p$$  \hspace{1cm} (7)

$$U_f = MCR_f \times [Glucose]_p$$  \hspace{1cm} (8)

To establish differences within tracer measures, ANOVA and Tukey post hoc test were employed. To determine differences over time for blood glucose, insulin, and NEFA, two-way repeated measures ANOVA was performed. Significance levels of $p \leq 0.05$ were used, and data are reported as means ± S.E.M.

**Results**

**Animal Characteristics.** The baseline characteristics of the animals fed CH and high-fat (HF) diets are summarized in Table 1. All measurements were done after a 10-h fast. The weight of the animals and their blood glucose levels did not differ between the two diets or treatment groups. HF feeding resulted in higher fasting NEFA and insulin levels compared with CH-fed animals ($p < 0.05$).

**Animal Experimentation.** Plasma glucose concentrations after infusions of saline and CVT-3619 are shown in Fig. 1. No differences between treatments for plasma glucose were noted at any time point. However, during the experimental period, plasma glucose increased steadily from baseline over time in all groups, regardless of diet or treatment ($p < 0.05$). Basal plasma glucose values (all groups) were 8.7 ± 0.3 mM at baseline compared with 10.4 ± 0.5 mM at the end of the experimental period. Results of plasma NEFA and insulin at baseline and after treatments are shown in Fig. 2. Saline infusion caused no change in NEFA compared with baseline in either CH or HF diet-fed animals (Fig. 2A); in comparison, CVT-3619 caused a 64% decline from baseline in NEFA in the HF diet-fed animals from baseline. Plasma insulin levels (Fig. 2B) showed no change with saline administration but declined with CVT-3619. In CH-fed rats, there was a small decrease in plasma insulin values that was not statistically significant ($p > 0.05$). However, CVT-3619 caused an 84% decrease in plasma NEFA concentrations relative to the concentration of nonradioactive cold substrate or tracee. Compared with CH, HF feeding resulted in a ~50% decrease in MCRg, which is indicative of an insulin-resistant state. These results were mirrored in the measurement of $U_p$ that showed a comparable decrease. Concordance between these two measures stems from the fact that blood glucose values remained fairly constant between groups throughout the experimental period. Administration of CVT-3619 increased both MCRg and $U_p$ in HF diet-fed rats, resulting in no difference in glucose kinetics between CH and HF diet groups treated with the drug ($p > 0.05$). The magnitude of this increase in glucose clearance with CVT-3619 in HF diet animals was intermediate between CH and HF control treatments. Analysis of fatty acid kinetics showed no difference in MCRf between treatments ($p > 0.05$). Likewise, no significant differences were noted for $U_f$.

**Cardiac Muscle Substrate Kinetics.** To examine substrate kinetics in the heart, indices of glucose and fatty acid clearance (K) and utilization (R) were calculated. Results for cardiac glucose and fatty acid clearance are shown in Fig. 3. Comparison of control and CVT-3619 administration in CH diet-fed animals revealed no difference in either glucose (Fig. 3, A and B) or fatty acid (Fig. 3, C and D) utilization (R) or clearance (K) between treatments ($p > 0.05$). Within HF, administration of CVT-3619 resulted in improved glucose clearance compared with controls ($p < 0.05$). These results are mimicked in the values for glucose utilization shown in

![Fig. 1. Plasma glucose at baseline (B) and during the experimental period in conscious rats treated with saline and CVT-3619 (n = 8 animals/treatment). No differences between treatments were noted at any individual time point as assessed by repeated measures ANOVA and a Tukey post hoc test ($p > 0.05$). Over time, plasma glucose increased from baseline to 30 min ($p < 0.05$) within all treatments. All data are reported as mean ± S.E.](image-url)
animals/treatment) as assessed by a repeated measures ANOVA and a glucose utilization. These findings are consistent with other perinsulinemia, hyperlipidemia, and reduced whole-body insulin-resistant phenotype as evidenced by fasting hy-

antilipolytic effects without cardiovascular, rebound, or de-
in adipose tissue (Fatholahi et al., 2006). CVT-3619 has analysis in a dose-dependent manner by reducing cAMP content in cardiac muscle in the fasting state is heavily dependent on fatty acids, with 60 to 90% energy derived from this source. Elevated circulating FFA combined with insulin resistance exacerbate this dependence on fatty acids, such that diabetic hearts are almost entirely dependent on fatty acids as a substrate (Lopaschuk et al., 1994). Ex vivo studies clearly demonstrate that cardiac fatty acid utilization is driven by circulating FFA levels. Employing the isolated perfused working heart model, Hafstad et al. (2006) reported that both control and leptin receptor-deficient (db/db) mouse hearts had increased rates of fatty acid utilization when perfused with a diabetic (high fat, high glucose) versus control (low fat, low glucose) perfusate. Such ex vivo data contrasts with in vivo studies (Oakes et al., 2006), which demonstrate that

Discussion

Insulin resistance results in myocardial dysfunction due to an oversupply of FFA that lead to changes in metabolism, gene expression, and contractile function (Murray et al., 2005; Carley et al., 2007). Reducing the availability and utilization of FFA is one therapeutic approach to normalize cardiac metabolism. In the present study, the acute effects of CVT-3619, a partial A1 adenosine receptor agonist, on whole-body and myocardial substrate kinetics were determined in diet-induced insulin-resistant rats. CVT-3619 inhibits lipolysis in a dose-dependent manner by reducing cAMP content in adipose tissue (Fatholahi et al., 2006). CVT-3619 has antilipolytic effects without cardiovascular, rebound, or desensitization effects normally associated with antilipolytic agents (Poynten et al., 2003; Dhall et al., 2007a).

HF diet feeding resulted in impaired glucose disposal and an insulin-resistant phenotype as evidenced by fasting hyperinsulinemia, hyperlipidemia, and reduced whole-body glucose utilization. These findings are consistent with other studies in rats using a similar HF diet and duration (Halseth et al., 2000; Reed et al., 2000). Acute administration of CVT-3619 caused a 64% decrease in plasma FFA in HF diet-fed animals. Employing glucose and fatty acid analogs to measure whole-body and cardiac substrate kinetics, results show that this decrease to be sufficient to alter substrate disposal (Fig. 3). On a whole-body level, administration of CVT-3619 did not significantly lower circulating FFA levels in CH diet-fed rats (Fig. 2) and therefore did not alter glucose or fatty acid substrate kinetics in these animals. In contrast, CVT-3619 did improve glucose clearance and utilization in HF diet-fed animals, suggesting the drug increases basal glucose disposal in an insulin-resistant state (Fig. 3). The expected reciprocal lowering of fatty acid utilization was not observed. This is likely because of the fact that insulin-resistant animals utilize a high proportion of FFA at rest (Hegarty et al., 2002). Of note, cardiac substrate utilization in control animals revealed no difference in glucose or fatty acid kinetics between CH and HF diet groups (Fig. 3). In contrast, administration of CVT-3619 increased glucose utilization and clearance in the heart of HF diet animals. Although this increase in clearance is disproportionately high, it results solely from the concomitantly decreases in FFA concentrations seen with the drug treatment. These findings confirm data of Dhall et al. (2007b), who showed that CVT-3619 administration for 2 weeks enhanced insulin action and lowered TG and plasma FFA levels in insulin-resistant rats. Likewise, the acute administration of CVT-3619 before a hyperinsulinemic-euglycemic clamp in mice also improved insulin sensitivity (Dhall et al., 2007b). Along with the present findings, data suggest that, by lowering FFA concentrations, CVT-3619 promotes cardiac glucose clearance and improves risk factors associated with insulin resistance.

The mechanisms by which CVT-3619 improves insulin sensitivity may involve both direct and indirect actions of the compound. Sharp declines in plasma insulin observed in HF-fed animals in the present study suggest a possible pancreatic effect of CVT-3619. The adenosine analog N6-phenylisopropyladenosine inhibits insulin secretion from the perfused pancreas by ~50% (Hillaire-Buys et al., 1987). Next, enhanced insulin sensitivity probably results from declines in circulating FFA concentrations (Fatholahi et al., 2006). By reducing cAMP formation, CVT-3619 inhibits lipolysis and thus lowers circulating FFA (Dhall et al., 2007b). This notion is consistent with the finding that overexpression of adipose A1 adenosine receptors in dietary-induced insulin-resistant mice lowers plasma FFA and improves insulin sensitivity (Dong et al., 2001). Of interest to the present study, cardiac muscle in the fasting state is heavily dependent on fatty acids, with 60 to 90% energy derived from this source.
anesthetized db/db mice do not have increases in fatty acid utilization in vivo but rather profound metabolic “inflexibility” (i.e., the inability to switch between substrates in the face of fluctuating substrate supply) (Oakes et al., 2006). Such metabolic inflexibility was not seen in the present study, presumably because HF diet-fed rats did not have overt type 2 diabetes but rather dietary-induced insulin resistance. There appears to be a discrepancy in the effects of type 2 diabetes on cardiac metabolism between in vivo and ex vivo models (Carley and Severson, 2008). For this reason, a conscious, unstressed in vivo animal model was employed in the present study. In vivo models maintain normal heart rhythm and replicate the range of circulating substrates available to the heart, including glucose, lactate, acetacetate, β-hydroxybutyrate, and fatty acids. When fatty acids, the substrates of choice were diminished in the present study, an increase in glucose uptake was observed in HF diet-fed animals. This shift to glucose utilization implies that fuel sensing remains intact in this model of diet-induced insulin resistance. The fact that this observation was only seen in HF diet-fed animals also suggests that their dependence on FFA was disproportionately high.

Cardiac metabolism and function are closely coupled. As such, normalizing circulating FFAs are also imperative for normal cardiac function. Surplus FFAs are detrimental in that they lead to altered gene expression, greater rates of fatty acid oxidation, greater oxygen demands, and lower cardiac efficiency (Mjös and Kjeekshus, 1971; Korvald et al., 2000; How et al., 2005). Circulating FFA concentrations are negatively correlated with ejection fraction in the chronically infarcted rat model (Murray et al., 2006). Furthermore, How et al. (2005) have demonstrated a 27% increase in oxygen consumption between perfusates containing low and high FFA in ex vivo mouse hearts. Of the excess oxygen consumed, differences in the phosphorylation to oxidation ratio between fatty acids and glucose could only account for 11% increased consumption in this study. Although greater oxygen was consumed, this did not translate to ATP production suggesting that increased fatty acid availability leads to mitochondrial uncoupling (Borst et al., 1962; Nabben and Hoeks, 2008). In essence, insulin resistance causes oxygen waste for noncontractile purposes resulting in overall reduced cardiac efficiency. Support of this hypothesis is derived from experiments showing uncoupling protein 3, a primary uncoupling protein in cardiac muscle, is positively correlated to heart failure (Vettor et al., 2002; Murray et al., 2004, 2008). Lastly, reducing plasma FFA levels by nicotinic acid abolishes the induction of uncoupling protein 3 (Van der Lee et al., 2001). Taken together, it is clear that elevated fatty acid concentrations increase oxygen consumption, promote mitochondrial

**TABLE 2**
Indices of whole-body glucose turnover in CH and HF diet-fed animals treated with either saline or CVT-3619

All data are reported as mean ± S.E. for the number of animals (n = 8) studied per treatment. MCRG and UG, along with MCRF and Uf are presented.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Treatment</th>
<th>MCRG</th>
<th>UG</th>
<th>MCRF</th>
<th>Uf</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH</td>
<td>Saline</td>
<td>1.2</td>
<td>0.1</td>
<td>10.7</td>
<td>2.3</td>
</tr>
<tr>
<td>CH</td>
<td>CVT-3619</td>
<td>1.3</td>
<td>0.3</td>
<td>11.1</td>
<td>2.5</td>
</tr>
<tr>
<td>HF</td>
<td>Saline</td>
<td>0.6</td>
<td>0.1</td>
<td>5.8</td>
<td>0.9</td>
</tr>
<tr>
<td>HF</td>
<td>CVT-3619</td>
<td>0.8</td>
<td>0.1</td>
<td>8.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* p < 0.05 between CH- and HF-fed animals within a treatment as assessed by an ANOVA and a Tukey post hoc test.
uncoupling, and lower cardiac efficiency. Such alterations are further exacerbated and are deleterious under ischemic conditions. Given this, it is not surprising that individuals with diabetes are at greater risk of an adverse outcome after a cardiac event (Miettinen et al., 1998).

In conclusion, acute administration of CVT-3619 results in an inhibition of lipolysis that lowers circulating plasma FFA and insulin levels in dietary-induced, insulin-resistant rats. As a result of these changes, basal rates of both whole-body and cardiac glucose utilization are improved. Taken together, these results show CVT-3619 to be a viable pharmaceutical tool for the manipulation of hyperglycemia, hyperinsulinemia, and perturbed patterns of substrate utilization in insulin resistance. Further studies are needed to determine the long-term effects of CVT-3619 and its efficacy in humans for the treatment of insulin resistance.

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References


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