Antilipolytic Activity of a Novel Partial A₁ Adenosine Receptor Agonist Devoid of Cardiovascular Effects: Comparison with Nicotinic Acid

Arvinder K. Dhalla, Melissa Santikul, Michelle Smith, Mei-Yee Wong, John C. Shryock, and Luiz Belardinelli

Department of Pharmacology, CV Therapeutics, Inc., Palo Alto, California

Received September 21, 2006; accepted January 2, 2007

ABSTRACT

Elevated lipolysis and circulating free fatty acid (FFA) levels have been linked to the pathogenesis of insulin resistance. A₁ adenosine receptor agonists are potent inhibitors of lipolysis. Several A₁ agonists have been tested as potential antilipolytic agents; however, their effect on the cardiovascular system remains a potential problem for development of these agents as drugs. In the present study, we report that CVT-3619 [(2-{6-[[((1R,2R)-2-hydroxycyclopentyl)amino]purin-9-yl}](4S,5S,2R,3R)-5-[(2-fluorophenylthio)methyl]oxolane-3,4-diol)], a novel partial A₁ receptor agonist, significantly reduces circulating FFA levels without any effect on heart rate and blood pressure in awake rats. Rats were implanted with indwelling arterial and venous cannulas to obtain serial blood samples, record arterial pressure, and administer drug. CVT-3619 decreased FFA levels in a dose-dependent manner at doses from 1 up to 10 mg/kg. The FFA-lowering effect was blocked by the A₁ receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine. Triglyceride (TG) levels were also significantly reduced by CVT-3619 treatment in the absence and presence of Triton. Tachyphylaxis of the antilipolytic effect of CVT-3619 (1 mg/kg i.v. bolus) was not observed with three consecutive treatments. An acute reduction of FFA by CVT-3619 was not followed by a rebound increase of FFA as seen with nicotinic acid. The potency of insulin to decrease lipolysis was increased 4-fold (p < 0.01) in the presence of CVT-3619 (0.5 mg/kg). In summary, CVT-3619 is an orally bioavailable A₁ agonist that lowers circulating FFA and TG levels by inhibiting lipolysis. CVT-3619 has antilipolytic effects at doses that do not elicit cardiovascular effects.

There is considerable experimental and clinical evidence that elevated levels of circulating free fatty acids (FFAs) play an important role in the pathogenesis of insulin resistance and diabetes (Chen et al., 1987; Reaven, 1995; Roden et al., 1996; Boden, 2001; Bays et al., 2004). Increases of adipose tissue mass and adipocyte cell size are associated with elevated blood FFA content. Enlarged adipocytes become resistant to the antilipolytic actions of insulin and release increased amounts of FFA into the circulation. Consequently, nonadipose tissues become exposed to elevated plasma FFA levels (Reaven, 1995; Boden, 2001; Bays et al., 2004). This results in increased deposition of triglycerides in peripheral tissues such as skeletal muscle, liver, pancreas, and heart, rendering these organs resistant to the actions of insulin (Sako and Grill, 1990; Roden et al., 1996; Itani et al., 2002; Boden et al., 2005). Therefore, because FFAs are central to the development of insulin resistance and lipid abnormalities of diabetes, reduction in elevated FFA levels is a goal in the treatment of insulin resistance and type 2 diabetes.

Despite overwhelming evidence of a role of elevated FFA in insulin resistance and diabetes, very few inhibitors of lipolysis are available for either experimental or clinical use. Nicotinic acid and its analog acipimox are the only well-characterized antilipolytic agents that are currently used for treatment of dyslipidemia (Carlson, 2005; Vega et al., 2005). Their therapeutic usefulness is limited because the initial decrease in plasma FFA levels is followed by a rebound that transiently increases FFA and insulin resistance (Poynten et al., 2003). Their therapeutic usefulness is limited because the initial decrease in plasma FFA levels is followed by a rebound that transiently increases FFA and insulin resistance (Poynten et al., 2003). Therefore, because FFAs are central to the development of insulin resistance and lipid abnormalities of diabetes, reduction in elevated FFA levels is a goal in the treatment of insulin resistance and type 2 diabetes.

ABBREVIATIONS: FFA, free fatty acid; TG, triglyceride; CVT-3619, (2-{6-[[[(1R,2R)-2-hydroxycyclopentyl]amino]purin-9-yl}](4S,5S,2R,3R)-5-[[2-fluorophenylthio)methyl]oxolane-3,4-diol); DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; CPA, N⁶-cyclopentyladenosine; PEG, polyethylene glycol; VLDL, very low-density lipoprotein; PIA, phenylisopropyladenosine.
ial usefulness in the treatment of insulin resistance and diabetes.

A₁ adenosine receptor agonists are well recognized antilipolytic agents due to their effect of reducing the formation and release of FFA from adipose tissue (Hoffman et al., 1986b; Gardner et al., 1994; Dhalla et al., 2003; Fraser et al., 2003; Schoelch et al., 2004; Fatholahi et al., 2006). A₁ agonists reduce lipolysis [breakdown of triglyceride (TG) to FFA] in adipose tissue by inhibiting adenyl cyclase activity and cAMP formation (Fain et al., 1972; Schwabe et al., 1974). The use and potential benefits of A₁ agonists to reduce lipolysis have been limited by the concurrent cardiovascular effects of this class of agents. The cardiac effects mediated by A₁ receptors include slowing of heart rate and atrioventricular nodal conduction, and depression of atrial contractility (Bellardinelli et al., 1989). However, due to a greater receptor reserve in adipose tissue compared with cardiac tissue (Wu et al., 2001; Liang et al., 2002), significant antilipolytic effects of A₁ agonists have been reported at doses that have either minimal or no cardiac effects (Gardner et al., 1994; Fraser et al., 2003). These findings suggest that it is possible to achieve organ selectivity for A₁ receptor-mediated responses. In this regard, partial agonists of the A₁ receptor may be useful to minimize unwanted cardiac effects because they elicit only submaximal responses in the heart even at high doses/concentrations (van Schaick et al., 1998; Wu et al., 2001).

CVT-3619, a derivative of adenosine, is a selective, partial agonist for the A₁ adenosine receptor that has been shown to inhibit lipolysis in isolated rat adipocytes at concentrations that do not have significant effects in isolated heart (Fatholahi et al., 2006). The present study was undertaken to characterize the antilipolytic and cardiovascular properties of CVT-3619 in vivo. The data show that CVT-3619 lowers plasma FFA and triglyceride levels in a dose-dependent manner without significant cardiovascular effects and also increases the potency of insulin as an antilipolytic hormone.

**Materials and Methods**

**Animals.** All experimental procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee of CV Therapeutics, Inc. and in accordance with the recommendations set forth in the Guide for the Care and Use of Laboratory Animals published by the National Research Council. Male Sprague-Dawley rats (300–325 g) with either one or two indwelling catheters (carotid and jugular) were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed 1 per cage in a room maintained on a 12 h light/dark cycle (lights on 6:00 AM to 6:00 PM) under constant temperature (22–25°C) with ad libitum access to food and water.

**Experimental Protocol.** The antilipolytic effects of CVT-3619 were studied in awake rats. Animals were fasted overnight before experimental use. On the day of the experiment, animals were put in metabolic cages and left undisturbed to acclimate to the environment for 1 to 2 h. An infusion set (21 gauge × 0.75 inches, 3.5 inches, 9-cm tubing, 0.17-ml volume) 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 FFA and TG. CVT-3619 was given via oral gavage, s.c. injection, i.v. injection, or i.p. injection as described for each different series of experiments. Blood samples were collected into serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) at predetermined times. Blood was allowed to clot and then centrifuged at 8000 rpm for 5 min at 4°C. The serum was stored at −80°C and was thawed at 4°C for determinations of FFA and TG contents.

**Cardiovascular Measurements.** The effects of CVT-3619 on heart rate and blood pressure were determined in a separate group of animals because heart rate is very easily affected in the unanesthetized animal by animal handling and blood sampling. Rats were instrumented with radiotelemetered transmitters (Data Sciences International, St. Paul, MN) at least 3 weeks before experimentation. The electrocardiogram, blood pressure, and temperature were recorded and heart rate calculated using a Dataquest ART Gold system (version 2.2; Data Sciences International). The system consisted of a transmitter, i.e., biopotential sensor (model TL11M2-C50-PXT), receivers (model RPC-1), a consolidation matrix (BCM 100), a personal computer (Compaq DeskPro Series 3574), and Dataquest 4 software. Heart rate, blood pressure and temperature were measured at 5-min intervals. Each recording lasted 10 s, and all cardiac cycles within this period were averaged.

**Chemicals and Reagents.** CVT-3619 was synthesized by the Department of Medicinal and Bioorganic Chemistry of CV Therapeutics, Inc. Sodium citrate, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), N⁶-cyclopentyladenosine (CPA), and Triton WR1339 were purchased from Sigma (St. Louis, MO). Nicotinic acid and PEG 400 were purchased from VWR (by EMD Biosciences, San Diego, CA). DPCPX was dissolved in 35% ethanol/65% water. CPA was dissolved in deionized water. Triton WR1339 was diluted in warm saline (~37°C) with frequent vortexing. Nicotinic acid was dissolved in saline. CVT-3619 was dissolved in PEG 400 by sonicating for 20 min and then diluted with distilled water to make a 20% PEG drug solution. FFA and TGs were measured using commercial kits from Wako Chemicals (Richmond, VA).

**Data Analysis.** All data are reported as mean ± S.E.M. Statistical analysis of data from experiments with two treatment groups was performed using the unpaired Student's t test. Two-way analysis of variance followed by Bonferroni's test was used for multiple comparisons. Differences among treatment groups were considered to be significant when the probability of their occurrence by chance alone was <0.05.

**Results**

**Effect of CVT-3619 on Plasma Free Fatty Acid and Triglyceride Levels.** CVT-3619 lowered FFA levels in a dose-dependent manner in normal, overnight-fasted awake rats. The time course of the effect of CVT-3619 on circulating serum FFA levels is shown in Fig. 1A. There was a small increase in FFA levels in the vehicle group at 10 min after the vehicle gavage. This response is probably due to an increase in lipolysis caused by the increase in sympathetic tone associated with the handling of awake animals. CVT-3619 at a dose of 2.5 mg/kg lowered FFA levels from 0.7 ± 0.05 to 0.5 ± 0.03 mM, a 31% decrease below baseline levels (p < 0.05). CVT-3619 lowered FFA levels by 47% to 0.4 ± 0.03 from 0.8 ± 0.04 mM at a dose of 5 mg/kg dose (p < 0.01). A 10 mg/kg dose caused a 57% decrease in FFA levels (from 0.68 ± 0.04 to 0.29 ± 0.02 mM, p < 0.001). The duration of the effect of CVT-3619 to suppress lipolysis was also dose-dependent (Fig. 1A).

To determine whether the A₁ receptor subtype was responsible for mediating the FFA-lowering effect of CVT-3619, rats were pretreated with DPCPX, an A₁ receptor antagonist, 10 min before administration of CVT-3619 (Fig. 1B). DPCPX (1

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3 For complete synthesis process for CVT-3619, refer to Elzein and Zablocki (2006a) and Elzein et al. (2006b).
mg/kg) itself caused a small increase in FFA levels. This is expected because DPCPX is not a neutral antagonist but instead an inverse agonist (Shryock et al., 1998). As clearly shown in Fig. 1B, pretreatment with DPCPX completely prevented the decrease in FFA caused by CVT-3619 (5 mg/kg).

CVT-3619 reduced serum triglyceride levels in a dose-dependent manner. The effect of three doses of CVT-3619 on serum triglycerides at 60 min post-treatment is shown in Fig. 2. TG levels were significantly decreased (\(p < 0.05\); \(p < 0.01\); \(p < 0.001\), indicating values that are significantly different from vehicle-treated group.

**Effect of CVT-3619 on Triglyceride Production.** To further investigate the mechanism of the decrease in TG levels by CVT-3619, total TG production was measured in normal rats. TG production was estimated by comparing the accumulation of TG in the plasma after an injection of Triton WR1339 (Triton, 600 mg/kg) in the absence and presence of CVT-3619 (Fig. 3). Treatment of rats with Triton induced a time-dependent increase in serum TG in both vehicle- and CVT-3619-treated rats. The increase in serum TG caused by Triton was significantly less in CVT-3619-treated animals compared with the vehicle-treated animals at 180 min post-treatment (\(p < 0.01\)). TG accumulation as determined from the slope of the line (linear regression analysis) was also significantly less (\(p < 0.001\)) in rats treated with CVT-3619 (3.8 ± 0.17 mg/dl/min) compared with vehicle-treated rats (5.6 ± 0.12 mg/dl/min).

**Lack of Tachyphylaxis to Repeated Treatment with CVT-3619.** The decrease in FFA levels caused by CVT-3619 was highly reproducible and did not undergo acute tachyphylaxis. As shown in Fig. 4, three repeated i.v. injections of CVT-3619 (1 mg/kg) to rats caused similar decreases in FFA levels to 0.35 ± 0.04, 0.35 ± 0.03, and 0.38 ± 0.03 mM, respectively, from a baseline value of 0.88 ± 0.02 mM. The time course of the decreases in plasma FFA levels caused by the three consecutive injections of CVT-3619 was similar.

**No Rebound with CVT-3619.** The antilipolytic effect of CVT-3619 was compared with that of nicotinic acid in overnight-fasted awake rats. CVT-3619 and nicotinic acid lowered FFA levels to 0.36 ± 0.05 from 0.79 ± 0.04 mM (\(p <
respectively (Fig. 5). CVT-3619 (1 mg/kg i.v. bolus) caused a maximal 54 ± 5% decrease in FFA levels that was comparable with that caused by nicotinic acid (57 ± 5%) given at a dose of 10 mg/kg i.v. bolus. The rebound increase of FFA levels seen with nicotinic acid was not observed with CVT-3619.

**Effect of CVT-3619 and Insulin on FFA Levels.** The effect of insulin (0.005–1 U/kg) to reduce serum FFA was determined in the absence and presence of a single dose (0.5 mg/kg) of CVT-3619 (Fig. 6). Baseline FFA levels in vehicle and CVT-3619 groups were 0.84 ± 0.01 and 0.92 ± 0.02 mM, respectively. CVT-3619 alone (0.5 mg/kg) caused an 18% decrease in FFA levels. As expected, insulin lowered FFA levels by up to 67 ± 1% in a dose-dependent manner. The doses of insulin that caused 50% decrease (ED50) in FFA levels in the absence and presence of CVT-3619 were 0.4 and 0.1 U/kg, respectively. Thus, in the presence of CVT-3619, there was a 4-fold leftward shift of the insulin dose-response to lower FFA, suggesting that CVT-3619 increases insulin sensitivity in adipose tissue.

**Cardiovascular Effects of CVT-3619.** The effect of CVT-3619 on heart rate and blood pressure was determined in awake rats by telemetry, and the data are shown in Fig. 7. CVT-3619 at doses of 1 and 5 mg/kg did not have a significant effect on heart rate but caused a small decrease (13 ± 1% calculated as area under the curve) in heart rate at a dose of 25 mg/kg (Fig. 7A). Increasing the dose of CVT-3619 to 50 mg/kg caused no further decrease in heart rate (data not shown). CVT-3619 did not have any significant effect on blood pressure at the doses used (Fig. 7B).

**Comparison between a Full A1 Agonist CPA and the Partial A1 Agonist CVT-3619.** The antilipolytic and hemodynamic effects of CVT-3619 were also compared with that of CPA, a full agonist of the A1 adenosine receptor. The results are shown in Fig. 8. CPA caused a 56% decrease from baseline in FFA levels at a dose of 20 μg/kg given via an i.p. injection (Fig. 8A). At the same dose, CPA caused significant bradycardia (from 387 to 187 bpm) that lasted for almost 30 min post-treatment (Fig. 8B). In contrast, CVT-3619 caused a similar decrease in FFA levels at a dose of 10 mg/kg (Fig. 8A) but had no significant effect on heart rate (Fig. 8B). A small transient increase in heart rate seen with CVT-3619 is probably due to increase in sympathetic tone caused by the handling of the rats and was also noted with vehicle treatment as shown in Fig. 7.
Discussion

High circulating FFA levels contribute to the development of insulin resistance and are considered a risk factor for type II diabetes and metabolic syndrome (Reaven, 1995). Adipose tissue lipolysis, which is highly regulated by insulin under normal conditions, is the major determinant of plasma FFA concentrations (Arner, 2005). However, the antilipolytic effect of insulin is impaired in insulin-resistant states, leading to an increased rate of lipolysis and high circulating FFA levels. Thus, antilipolytic agents that can normalize the rate of lipolysis in insulin-resistant states should be clinically useful.

Recently, we have shown that CVT-3619 is a selective and partial A₁ adenosine receptor agonist, with antilipolytic activity (Fatholahi et al., 2006). CVT-3619 inhibited cAMP accumulation and FFA release from rat adipocytes and had minimal effects on cardiac function in isolated heart preparation (Fatholahi et al., 2006). The present study investigated the in vivo metabolic and cardiovascular effects of CVT-3619 in awake rats. The results show that CVT-3619 lowers FFA and TG in a dose-dependent manner. The FFA-lowering effect does not undergo tachyphylaxis and is not associated with a rebound. The antilipolytic effects occur at doses that have no significant cardiovascular effects. The antilipolytic effects of CVT-3619 were also compared with that of nicotinic acid, a potent and clinically used antilipolytic agent. Last, but importantly, CVT-3619 increased the potency of insulin to reduce plasma FFA concentrations.

CVT-3619 lowered circulating FFA levels in a dose-dependent manner. These data are consistent with previous reports showing that other A₁ agonists decrease FFA levels (Hoffman et al., 1986b; Gardner et al., 1994; Fraser et al., 2003; Schoelch et al., 2004). The FFA-lowering effect of CVT-3619 was completely antagonized by pretreatment with an A₁ antagonist, DPCPX, confirming that these effects are mediated via A₁ receptors. CVT-3619 also caused a significant decrease in TG levels. The decrease in TG secretion is probably due to decreased substrate (FFA) availability in the liver, as has been shown previously (Hoffman et al., 1986b; Gardner et al., 1994). Limiting the supply of FFA to the liver decreases the output of triacylglycerol (VLDL) and ketone bodies, thus producing both hypotriglyceridemic and antiketotic effects (Kovoor et al., 1998). Although we did not measure VLDL production, the decrease in TG production by CVT-3619 is also expected to result in decreased VLDL production as previously shown using R-phenylisopropyladenosine (PIA) (Hoffman et al., 1986b).

Tachyphylaxis and receptor desensitization are potential problems when considering a receptor agonist as a drug for long-term use. It has been shown that A₁ receptors undergo agonist-induced long-term desensitization but are not subject to rapid acute desensitization (Gao et al., 1999). The antilipolytic effects of CVT-3619 were well maintained over three consecutive administrations. The magnitude and the duration of the FFA-lowering effect of CVT-3619 were similar for all three injections, suggesting that the effect of this agonist does not undergo tachyphylaxis. Desensitization of the antilipolytic effect of A₁ receptors has been shown to occur with prolonged and continuous exposure to high concentrations of an A₁ agonist, R-PIA (Hoffman et al., 1986a). R-PIA is a full agonist and thus more likely to cause desensitization. In contrast to R-PIA, CVT-3619 is a partial A₁ receptor agonist. Partial agonists of GPCRs have been suggested to cause less...
Adenosine analogs. The differential sensitivity to A1 agonists is limited by side effects such as flushing and a post-treatment rebound increase in FFA (McKenny et al., 1994). The suppression of lipolysis by nicotinic acid is followed by a rebound in FFA release, such that the levels of FFA rise above the baseline upon washout of the effect (Pereira, 1967; Blackard and Heidingsfelder, 1969). The rebound has been suggested to be responsible for the paradoxical decrease in insulin sensitivity observed when using large doses of nicotinic acid (Kelly et al., 2000; Poynten et al., 2003). The mechanism of FFA rebound with nicotinic acid remains unknown. It has been suggested that the magnitude of rebound is dependent upon the magnitude of decrease in FFA, and a significant correlation between FFA lowering and rebound has been shown for nicotinic acid (Blackard and Heidingsfelder, 1969; Schwabe et al., 1974). Rebound increase in plasma FFA levels was not observed with CVT-3619, even though FFA concentrations were decreased by similar extent by both CVT-3619 and nicotinic acid, i.e., 54 and 57% from baseline, respectively. It has been shown that the FFA rebound still exists with an extended-release nicotinic acid formulation (Vega et al., 2005), which suggests that the rebound phenomenon may be unique to nicotinic acid and may not apply to other antilipolytic agents.

The antilipolytic actions of adenosine that are mediated by A1 receptors have been reported for many years (Hoffman et al., 1986b; Gardner et al., 1994; Fraser et al., 2003; Schoelch et al., 2004). The metabolic responses after acute administration of many A1 agonists have been reported previously; however, no compound has been developed and approved for clinical use thus far. A possible reason for this is lack of separation between the cardiac (and perhaps central nervous system) and the antilipolytic effects (Dhalla et al., 2003). It is possible, however, to achieve functional selectivity using partial agonists as described previously (van Schaik et al., 1998; Wu et al., 2001). The position of the dose- or concentration-response relationship for the antilipolytic and cardiovascular effects of CVT-3619 are further apart than for full A1 receptor agonists such as CPA (Fig. 8). CPA caused marked bradycardia at a dose (i.e., 20 μg/kg) that caused a similar decrease in FFA levels as that observed with CVT-3619 at 10 mg/kg, which had no effect on heart rate. Although some degree of functional selectivity can also be achieved with full agonists (Fraser et al., 2003), the difference between the effective dose to lower FFA and to depress cardiac function is greater for partial than full agonists, making them much safer drugs. Functional selectivity of CVT-3619 to decrease lipolysis and to lower FFA levels relative to heart rate was greater than 25-fold (compare Fig. 1 with 7). This differential response to CVT-3619 results from the much higher sensitivity of adipose tissue (compared with cardiac tissue) to adenosine analogs. The differential sensitivity to A1 agonists has been explained on the basis of the differences in the receptor reserve in the two tissues (Liang et al., 2002; Fatholahii et al., 2006). The possibility of the existence of different receptors in the heart and adipose tissue has been ruled out previously (Tatsis-Kotsidis and Erlanger, 1999; Fatholahii et al., 2006).

Adenosine has been shown to modulate insulin actions and insulin sensitivity in muscle and adipose tissue (Budohoski et al., 1984; Rolband et al., 1990). In adipocytes, the increase in insulin sensitivity by adenosine was suggested to be mediated by A1 receptors. PLA, an A1 adenosine receptor agonist, potentiated the insulin-induced activation of PI3 kinase, a second messenger for insulin actions, in rat adipocytes (Takasuga et al., 1999). Our data show that in the presence of CVT-3619, the ED50 for insulin to inhibit lipolysis in vivo is decreased 4-fold, suggesting that CVT-3619 increases insulin sensitivity in adipose tissue. This potentiation of the FFA-lowering effect occurs at a much lower dose of CVT-3169 (0.5 mg/kg) than those used for investigating the antilipolytic effects of CVT-3619 alone (1.0 mg/kg and higher). Thus, CVT-3619 could be useful in insulin-resistant states where antilipolytic effect of insulin is impaired, and the rate of lipolysis is increased, leading to high circulating levels of FFA.

In conclusion, data in the present study show that CVT-3619, an A1 adenosine receptor partial agonist, is an effective antilipolytic agent that lowers circulating FFA and TG levels and improves insulin sensitivity in adipose tissue. The antilipolytic effect of CVT-3619 is not associated with a rebound increase FFA. The FFA-lowering effects occur at doses that have no effect on heart rate and blood pressure. The pharmacological properties of CVT-3619 suggest that this compound may have therapeutic utility in metabolic and cardiovascular disorders in which FFA levels are increased.

References
antilipolytic effects of partial a1 adenosine receptor agonist


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