Acipimox stimulates leptin production from isolated rat adipocytes

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Abstract
Acipimox is a nicotinic acid-derived antilipolytic drug devoid of major side effects, and has been used in a number of human trials. This work reports the effects of Acipimox on leptin production from isolated rat adipocytes, in comparison with nicotinic acid and insulin. For cells isolated from normal animals, all these three reagents stimulated leptin release to a similar extent. Acipimox and nicotinic acid were more potent than insulin in stimulating leptin release from cells isolated from diabetic animals, probably because of impaired insulin sensitivity in cells from these diseased animals. Co-incubation of Acipimox with norepinephrine or dibutyryl cAMP diminished its stimulatory effects on leptin release, in parallel with increased lipolysis, suggesting that intracellular free fatty acids play an important role in mediating leptin production in adipocytes.


Introduction
Being a satiety hormone predominantly produced from adipose tissue, leptin has been proposed to play important roles in regulating body energy balance. The impairment of this energy balance leads to obesity, which is becoming a prevailing disease that affects the whole world. Obesity is associated with elevated plasma free fatty acid (FFA) concentrations caused by impaired insulin suppression of adipocyte lipolysis. Some prior studies suggest that FFA or their acyl CoA products inhibit leptin production (Rentsch & Chiesi 1996, Deng et al. 1997, Shintani et al. 2000), whereas others show that decrease in plasma FFA caused by infusion of Acipimox does not change plasma leptin concentrations in the short term (Hennes et al. 1997, Peino et al. 1998, Stumvoll et al. 2000). In a recent study, a 3-day treatment with Acipimox was found to lower plasma FFA, triglycerides (TG), glucose, and insulin, and also significantly increase plasma leptin level (Worm 2000 et al.). Because insulin stimulation on leptin production has been well documented both in vivo and in vitro, increased leptin levels induced by Acipimox treatment is not likely to be a secondary effect of decreased insulin production. It suggests that Acipimox may have a direct effect on leptin production. The discrepancy between the long-term and short-term studies indicates the effect of Acipimox on leptin production may be a chronic effect. Alternatively, since leptin production is regulated by various factors in vivo, the effects of Acipimox may not become clear until other factors are coordinately regulated. In this regard, an in vitro system may be valuable; for it allows an investigation of extrinsic factor(s) in the absence of homeostatic compensatory mechanisms commonly found in vivo.

Isolated primary adipocytes have been widely used as an in vitro model system to study individual factors that influence cell functions, including leptin production (Mueller et al. 1998, Shintani et al. 2000, Wang et al. 2002). In this work, we investigated how Acipimox modulates leptin secretion and other metabolic events in freshly isolated rat adipocytes from normal, streptozotocin (STZ)-diabetic, Zucker diabetic fat (ZDF) and Zucker lean (Z-lean) rats. Our results demonstrated a close similarity between Acipimox and insulin on their effects on leptin production from cells isolated from normal animals. However, Acipimox is more potent than insulin in stimulating leptin production from cells isolated from diabetic rats.

Material and Methods
Chemicals
Acipimox (5-methyl-pyrazine-2-carboxylic acid-4-oxide) was obtained from Pharmacia (Spa, Italy). Collagenase (type II), BSA (fraction V), STZ, insulin powder (porcine), nicotinic acid, dibutyryl cAMP (Bt2-cAMP), norepinephrine (NE) bitartrate and other chemicals were purchased from Sigma Co. (St Louis, MO, USA). Rat leptin RIA kit was purchased from Linco Research Inc. (St Charles, MO, USA).

Animals
Normal female Sprague–Dawley rats (280–300 g) were purchased from Charles River Laboratory (Wilmington,
MA, USA). STZ–diabetic rats were prepared by peritoneal injection of STZ 50–60 mg/kg. After 3–5 days of injection, blood glucose and urinary glucose levels were tested to confirm diabetic status and rats were ready for use. ZDF rats (290–330 g, fa/fa) and Z-lean rats (280–300 g) were purchased from Genetic Models Inc. Indianapolis, IN, USA. All animals were housed with free access to laboratory chow and tap water, with a photoperiod of 12 h light:12 h darkness at 26 °C. The plasma leptin, insulin, glucose and TG levels of these animals at the time of experiments are listed in Table 1.

**Table 1** The plasma parameters (n=6, means ± S.E.M.) in non-fasting rats at the time of experiments

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>STZ-diabetic</th>
<th>Z-lean</th>
<th>ZDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (ng/ml)</td>
<td>19.5±5</td>
<td>0.7±0.1</td>
<td>14.5±0.9</td>
<td>29.1±0.9</td>
</tr>
<tr>
<td>Glucose (mg/ml)</td>
<td>109±4.1</td>
<td>744±83</td>
<td>113±6.2</td>
<td>289±24</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>1.3±0.3</td>
<td>0.5±0.02</td>
<td>6.7±0.9</td>
<td>24.6±1.7</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>62±3</td>
<td>194±9</td>
<td>200±16</td>
<td>511±25</td>
</tr>
</tbody>
</table>

Adipocyte preparation

White adipocytes were prepared from perirenal fat pads of non-fasting animals by collagenase digestion with Rodbell’s method with slight modification as described before (Rodbell 1967, Wang et al. 2002). The isolated adipocytes were finally washed with 5 ml Krebs–Ringer bicarbonate (KRB) containing 4% BSA and diluted at a concentration of 0.15–0.25 × 10⁶ cells/ml in KRB (glucose 2.7 mM, BSA 4%, pH 7.4). The cell numbers were counted in a hemacytometer after trypan blue staining.

Leptin and glycerol release

Isolated adipocytes were incubated with insulin, Acipimox or nicotinic acid alone for up to 4 h at 37 °C with shaking in a water bath at 40 cycles/min. NE or Bt2-cAMP was added to selected incubations together with Acipimox. The release of leptin or glycerol was determined as described before (Wang et al. 2002).

Statistical analysis

StatView II, DeltaGraph software was used to process the data (F-test). Results are shown as means ± S.E.M.

Results

Acipimox-stimulated leptin secretion in adipocytes from normal rats

While the acute effects of Acipimox on leptin production were not detected in vivo (Stumvoll et al. 2000), its stimulation was rapidly detected in vitro. As shown in Fig. 1A, within 2 h of incubation, there was about 50% increase in leptin release from cells incubated with Acipimox as compared with the control, in agreement with the rapid time course of drug-induced changes in leptin expression reported before (Chen et al. 1999). Nicotinic acid caused a similar level of stimulation on leptin release under identical conditions. Both demonstrated an insulin mimicking effect (Fig. 1A). At the end of 4 h of incubation, leptin release from the treated cells was more than 2-fold of the control (Fig. 1A). Figure 1B shows the dose-dependent stimulation of Acipimox on leptin release. Below 10⁻⁵ M, Acipimox showed essentially no effect on leptin release. From 10⁻⁵ M to 10⁻³ M, there was a rapid increase in leptin release as a function of Acipimox concentration and this effect was saturated beyond 10⁻³ M. The dose–response pattern was the same for Acipimox/nicotinic acid (10–100 mM, EC₅₀ ~ 0.5 mM) and resembled that of insulin (0.001–100 nM, EC₅₀ ~ 1 nM). Maximal stimulation was higher for Acipimox/nicotinic acid than insulin (3.85 vs 3.25 ng/10⁶ cells, P<0.05).

Acipimox-stimulated leptin release in adipocytes from diabetic rats

Adipose tissue leptin mRNA and plasma leptin level have been found to decrease in insulin-deficient (type 1) diabetic donors (Sivitz et al. 1996). Treatment by insulin restored the leptin production in vivo (Sivitz et al. 1996, Ahmed et al. 2001). To test whether this can be a direct effect of insulin on adipocytes and whether such actions can be mimicked by Acipimox, effects of these agents on leptin production from adipocytes of STZ-diabetic rats were determined. As shown in Fig. 2, incubation with insulin or Acipimox significantly increased leptin release. However, insulin stimulation was clearly less potent on cells from the STZ animals than those from the control, whereas Acipimox had a similar stimulation on both (Fig. 2). These results are consistent with the previous study that shows adipocytes of STZ–diabetic rats had reduced sensitivity to insulin (Nishimura et al. 1989). We next looked into the animal model of type 2 diabetes, the ZDF rats. Similar to the results shown above, basal leptin release was similar from adipocytes from Z-lean and ZDF.
rats (Fig. 2). Both insulin and Acipimox stimulated leptin release (Fig. 2). However, insulin stimulation was less potent for ZDF adipocytes than for the Z-lean, whereas Acipimox stimulation was almost the same for adipocytes of both types of rats.

Note that adipocytes from both types of diabetic rats have similar basal leptin release as control (Fig. 2), in contrast to the large difference found in the corresponding plasma leptin concentrations (Table 1). This discrepancy is probably caused by the difference in the hormonal and nutritional conditions in vivo and in vitro as described before (Wang et al. 2002).

NE inhibits Acipimox-stimulated leptin release

NE has been shown to diminish insulin-stimulated leptin release in rat adipocytes (Gettys et al. 1996). To determine whether it also affects the stimulatory effects of Acipimox, adipocytes from Sprague–Dawley rats were incubated with NE and Acipimox at various concentrations. As shown in Fig. 3, at fixed NE concentration (10⁻⁵ M), increase of Acipimox stimulated leptin release dose-dependently. However, at any given concentration of Acipimox, the presence of NE largely reduced the leptin release compared with that in the absence of NE (Fig. 3 vs Fig. 1B). At a sub-optimal concentration of Acipimox (10⁻³ M), its stimulation on leptin release was attenuated with increasing NE concentrations (Fig. 3, IC₅₀ ~0·25 µM). This is similar to a prior report that NE inhibits insulin-stimulated leptin release (IC₅₀ ~0·1 µM).
The NE-mediated decrease in leptin release was accompanied with a simultaneous increase in glycerol release (Fig. 3). Co-incubation of adipocytes with Bt2-cAMP and Acipimox also diminishes the stimulatory effects of Acipimox on leptin release in a similar pattern as NE (Fig. 4), consistent with prior reports that increased intracellular cAMP is associated with decreased leptin production (Gettys et al. 1996, Kosaki et al. 1996, Slieker et al. 1996).

Discussion

This study is, to our knowledge, the first report that Acipimox, similar to insulin, acutely stimulated the leptin release in isolated primary rat adipocytes. Moreover, we showed that while insulin-stimulated leptin production was impaired for cells from diabetic animals, the stimulatory effect of Acipimox was essentially not affected (Fig. 2). Like some other insulin mimicking reagents, Acipimox may bypass the membrane receptor control to reach the target regulatory machinery via alternative pathways.

Previous studies suggest that acute regulation of leptin production by β-adrenoceptor agonists is mediated via the second messenger cAMP (Kosaki et al. 1996, Deng et al. 1997, Donahoo et al. 1997, Coya et al. 2001), although an alternative cAMP-independent signal pathway may be involved (Stumvoll et al. 2000). Nevertheless, our data showed that Acipimox stimulation was efficiently suppressed by both NE and Bt2-cAMP, consistent with the hypothesis that Acipimox acts mainly via its inhibition on cAMP production. Since the ultimate result of increased cAMP is the rise in FFA release, the intracellular FFA level may play key roles in this process (Sivitz 1996, Havel et al. 1998, Shintani et al. 2000). Indeed, in cultured 3T3-L1 adipocytes, incubation with FFA exerts a concentration-dependent inhibition of leptin transcription whereas an elevation of cAMP alone displayed only a marginal effect (Rentsch & Chiesi 1996).

The molecular mechanisms that control leptin production are still not well understood. It has been shown that the adipogenic transcription factor C/EBPα binds to the leptin promoter and activates its transcription (He et al. 1995, de la Brousse et al. 1996, Hwang et al. 1996). Although direct interaction between FFA and C/EBPα has not been established, indirect interactions may be mediated by peroxisome proliferator-activated receptor-γ (PPAR-γ, the ligand-activated adipocyte specific nuclear transcription factor (Hamm et al. 2001)). Activation of PPAR-γ by its pharmacological ligand suppresses the transcription of leptin gene (De Vos et al. 1996, Kallen & Lazar 1996, Nolan et al. 1996). FFAs have been shown to bind and activate PPAR-γ in vitro (Kliewer et al. 1997), and may act as endogenous ligands for this transcription factor (Grimaldi et al. 1999). The suppression of leptin gene expression by elevated FFA might be mediated via the activation of PPAR-γ (Shintani et al. 2000). In this regard, Acipimox-stimulated leptin production could be caused by FFA-mediated changes in PPAR-γ activity.

A few recent studies show that leptin induces lipolysis in adipocytes (Wang et al. 1999, Kawaji et al. 2001, Yamagishi et al. 2001). Meanwhile, exogenous leptin also increases the expression and enzyme activities of carnitine palmitoyl transferase-1 (CPT-1) and decreases that of acetyl CoA carboxylase (ACC), the two key enzymes that control FFA oxidation (Wang et al. 1999, Yamagishi et al. 2001).
Acipimox and adipocyte leptin production

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Acipimox β-agonist

Leptin

CPT-I, ACO

ACC

FAS

PPARγ

FFA oxidation

Figure 5 A schematic model of the leptin–FFA regulatory loop. Solid lines indicate stimulation, whereas dashed lines indicate inhibition. ACC, acetyl CoA carboxylase; ACO, acyl CoA oxidase; CPT-I, carnitine palmitoyl transferase-1; FAS, fatty acid synthase.

2001). It should be noted that CPT-1 and acyl CoA oxidase (ACO) as well as their transcription factor, PPAR-α, are regulated by FFA (Sleboda et al. 1999, Geelen et al. 2001). Therefore, leptin-induced activation of β-oxidation might be actually mediated by leptin-stimulated lipolytic FFA release. When increased β-oxidation can no longer accommodate the rise of intracellular FFA release, endogenous leptin production is inhibited. Such a leptin–FFA regulatory loop is schematically summarized in Fig. 5.

Under our experimental conditions, inhibition on leptin production by FFA could have become significant after the adipose tissue was dissected and processed in the KRB buffer, with a moderately low glucose concentration (2–7 mM), high BSA concentration and no insulin, the conditions resembling starvation. In this case, basal FFA release was relatively high (data not shown). Adding Acipimox to this system inhibited FFA release and removed its inhibition on leptin production, leading to increased leptin release (Fig. 1). Our model does not seem to agree with a prior report that shows no effect on plasma leptin concentration caused by acute changes in plasma FFA in vivo (Peino et al. 1998). However, in this study, plasma FFA was elevated by i.v. infusion of intralipid with heparin. In this case, the intracellular FFA in adipocytes might not be significantly affected. Alternatively, changes of various compensatory factors in vivo may mask its effects on leptin production (Leal-Cerro et al. 1997).

In summary, although circulating leptin level is usually increased in obesity, leptin production per equivalent unit of fat mass is reduced (Surwit et al. 1997, Watson et al. 2000). Moreover, hyperinsulinemia commonly found in obese subjects causes elevated basal leptin levels but diminishes hormone-stimulated leptin expression, and diminishes the metabolic regulatory functions of leptin. Our findings that Acipimox-stimulated leptin production is not impaired by diabetes-induced receptor malfunctioning warrant further investigation on this drug for the regulation of fat mass development.

Acknowledgement

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