Evidence for fatty acids mediating CL 316,243-induced reductions in blood glucose in mice

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MacPherson RE, Castellani L, Beaudoin M, Wright DC. Evidence for fatty acids mediating CL 316,243-induced reductions in blood glucose in mice. Am J Physiol Endocrinol Metab 307: E563–E570, 2014. First published August 5, 2014; doi:10.1152/ajpendo.00287.2014.—CL 316,243, a β3-adrenergic agonist, was developed as an antiobesity and diabetes drug and causes rapid decreases in blood glucose levels in mice. The mechanisms mediating this effect have not been fully elucidated; thus, the purpose of the current study was to examine the role of fatty acids and interleukin-6, reputed mediators of insulin secretion, in this process. To address this question, we used physiological and pharmacological approaches in combination with knock-out mouse models. CL 316,243 treatment in male C57BL/6J mice increased plasma fatty acids, glycerol, interleukin-6, and insulin and reduced blood glucose concentrations 2 h following injections. The ability of CL 316,243 to increase insulin and fatty acids and reduce glucose was preserved in interleukin-6-deficient mice. CL 316,243-induced drops in blood glucose occurred in parallel with increases in circulating fatty acids but prior to increases in plasma interleukin-6. CL 316,243-mediated increases in plasma insulin levels and reductions in blood glucose were attenuated when mice were pretreated with the lipase inhibitor nicotinic acid or in whole body adipose tissue triglyceride lipase knockout mice. Collectively, our findings demonstrate an important role for fatty acids in mediating the effects of CL 316,243 in mice. Not only do our results provide new insight into the mechanisms of action of CL 316,243, but they also hint at an unappreciated aspect of adipose tissue -pancreas cross-talk.

CL 316,243 (CL) is a specific β3-adrenergic agonist originally developed as an antiobesity and antiadipotic drug (1). Treating obese, insulin-resistant rodents with this compound for several weeks leads to improvements in glucose homeostasis and insulin sensitivity (7, 8, 31) that are likely related to CL-mediated decreases in food intake (7, 9), increases in energy expenditure (9), and subsequent reductions in adipose tissue mass (7, 8, 31). In contrast, in lean human subjects, CL treatment results in increases in insulin-mediated glucose disposal (36) independent of changes in body composition, suggesting a direct effect of this compound on insulin sensitivity. In addition to these longer-term effects, CL rapidly (i.e., within minutes) increases insulin secretion and reduces blood glucose levels in mice in vivo (9). The whole body deletion of β3-adrenergic receptors abolishes CL-mediated reductions in blood glucose and this is prevented when β3-adrenergic receptors are reintroduced into white and brown adipocytes (9). However, when β3-adrenergic receptors are reintroduced into brown adipocytes only, the glucose lowering effects of CL are not recovered (9), providing evidence that a factor secreted from white adipocytes mediates the effects of CL on insulin secretion and reductions in blood glucose. At this point, the specific factor(s) mediating this pronounced glucose lowering effect is not known. Delineating these mechanisms will provide new insights into adipose tissue and integrative physiology with relevance to the development of approaches to lower blood glucose.

In recent work from our laboratory, we have shown that the mRNA expression of the cytokine, IL-6 (interleukin-6), is increased ~100-fold in epididymal adipose tissue following a single injection of CL, and this is mirrored by ~7-fold increases in circulating IL-6 (3). Although IL-6 can also be secreted from other tissues, the high expression of β3-adrenergic receptors in white adipocytes (19) and the robust induction of IL-6 in white adipose tissue with CL suggest that adipocytes are the source of IL-6. IL-6 has been shown to increase insulin secretion in cultured islets (24, 28), while more recent work has reported that IL-6 potentiates glucose-stimulated insulin secretion in vivo through the incretin hormone GLP-1 (glucagon-like peptide-1) (6). These findings provide evidence that IL-6 could be involved in CL-induced reductions in blood glucose levels.

In addition to increasing circulating IL-6 levels, CL treatment also causes large increases in plasma fatty acids (9, 23). Fatty acids such as palmitate have been shown to potentiate glucose stimulated insulin secretion in isolated rodent islets (15, 21), and in fasted rats (29). Similarly, experimentally increasing plasma fatty acids to supraphysiologigic levels (26) increases insulin secretion and lowers blood glucose, whereas physiological increases in plasma fatty acids result in much more subtle changes in these end points in dogs (5, 26). Of interest, the effects of CL on the induction of IL-6 in adipose tissue is blunted when fatty acid release is pharmacologically attenuated in vivo (18) and would suggest that the potential glucose lowering effects of fatty acids with CL treatment could be mediated, at least in part, through increases in IL-6.

The purpose of the current investigation was to examine the role of fatty acids and IL-6 in mediating CL-induced increases in plasma insulin and reductions in blood glucose in mice in vivo. Specifically, we hypothesized that increases in insulin secretion and reductions in blood glucose by CL would be attenuated when increases in fatty acids or IL-6 were blocked. To address this supposition, we utilized physiological and pharmacological approaches in combination with genetically modified mice.

**MATERIALS AND METHODS**

**Materials**

CL 316, 243 (cat. no. C5976) and free glycerol assay kits (cat. no. FG0100) were purchased from Sigma Aldrich (Oakville, ON, Canada). ELISAs for IL-6 (cat. no. EZMIL6) and insulin (cat. no. 0193-1849/14 Copyright © 2014 the American Physiological Society
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CL 316,243-INDUCED REDUCTIONS IN BLOOD GLUCOSE IN MICE

EZRMI-13K) were obtained from Millipore (Billerica, MA). Nones-
terified free fatty acid (NEFA) kits were from Wako Chemicals
(Richmond, VA). Oligo(dt), SuperScript II Reverse Transcriptase,
and dNTP were from Invitrogen (Burlington, ON, Canada). Taqman
gene expression assays for IL-6 (Mn00446190_m1) and GAPDH
(4352932E) were from Applied Biosystems (Foster City, CA). All
other materials were purchased from Sigma.

Treatment of Animals

Approximately 10-wk-old male C57BL/6 mice were purchased
from Charles River, while 10 wk-old male IL-6−/− (B6.12952-
IL6tmkopf/J), ATGL−/− (B6;129P2-Pppla2tm18orj) and age-matched
wild-type (WT) control (C57BL/6/J) mice were obtained from Jackson
Labs. Animals were housed one per cage, had free access to water and
standard rodent chow, and were maintained on a 12:12-h light-dark
cycle. All protocols were approved by the University of Guelph
Animal Care Committee and met the Canadian Council on Animal
Care (CCAC) guidelines.

CL 316,243 treatment. In the fed state, at ~9:00 AM, mice were
injected with a weight adjusted (1 mg/kg body wt ip) bolus of the
β3-adrenergic agonist CL 316,243 (CL) or an equivalent volume of
sterile saline. We chose this dose of CL because previous studies had
reported the induction of IL-6 mRNA expression in adipose tissue (2, 23)
and a lowering of blood glucose using a similar dosage (9). Two
hours following CL treatment, mice were anesthetized with pentobar-
bital sodium (5 mg/100 g body wt), and blood glucose was
determined through tail vein blood with a hand-held glucometer.
Epididymal adipose tissue was harvested and intrathoracic blood was
collected. To ascertain the temporal relationship between increases
in plasma IL-6 and reductions in blood glucose, we repeated these
experiments and harvested tissue 15 min post-CL treatment.

CL 316,243 treatment in IL-6 deficient mice. To ascertain the
involvement of IL-6 in the glucose lowering effects of CL, male WT
and whole body knockout IL-6 deficient mice were injected with CL
(1.0 mg/kg body wt) or an equivalent volume of sterile saline, and
changes in blood glucose were determined 30, 60, 90, and 120 min
postinjection from the tail vein with a hand-held glucometer. Mice
were then anesthetized, and thoracic blood was collected for determi-
nation of terminal insulin, IL-6, fatty acids, and glycerol.

CL 316,243 treatment in nicotinic acid-treated mice. Mice were
treated with nicotinic acid (250 mg/kg body wt ip) or an equivalent
volume of sterile saline 15 min prior to treatment with CL. Blood
glucose was determined prior to and 30 min following CL treatment.
Blood from the saphenous vein was collected prior to and 15 min after
injection for the analysis of plasma insulin and fatty acid levels. We
chose this shorter time course because we were concerned that the
pharmacological activation of lipolysis with CL could override the
inhibitory effects of nicotinic acid.

CL 316,243 treatment in ATGL knockout mice. Whole body male
ATGL-deficient mice or WT controls were injected with CL, and
saphenous blood was collected pre- and 30 min postinjection for the
determination of fatty acids and insulin. Blood glucose was measured
at the same time points from the tail vein with a glucometer. Mice
were then anesthetized, and thoracic blood was collected for the
determination of IL-6.

Plasma Glycerol, Fatty Acids, Insulin and IL-6

Analysis of metabolite and hormone concentrations was deter-
mined using commercially available kits, following the manufactur-
er’s instructions. Samples were run in duplicate and the average CV
was <10%. All samples from one experiment were run on the same
plate.

Real-Time PCR

Changes in mRNA expression of IL-6 were determined using
real-time qPCR as described in detail previously by our laboratory
(32, 33). RNA was isolated from epididymal white adipose tissue
using an RNeasy kit according to the manufacturer’s instructions.
Complementary DNA (cDNA) was synthesized from total RNA (1 μg)
using SuperScript II Reverse Transcriptase, dNTP, and oligo(dt).
Real-time PCR was completed using a 7500 Fast Real-Time PCR
system (Applied Biosystems). Samples were run in duplicate in a
96-well plate. Each well contained a total volume of 20 μl with 1 μl
gene expression reagents, 1 μl cDNA template, 10 μl Taqman Fast
Universal PCR Master Mix, and 8 μl RNase-free water. Changes in
mRNA expression were expressed relative to the expression of
GAPDH, and differences in gene expression between saline and CL
treated mice were determined using the 2−ΔΔCT method (17). The
PCR efficiency was similar between IL-6 and GAPDH.

Statistical Analysis

Comparisons between groups were made using unpaired, two-
tailed, t-tests. Differences between IL-6-deficient and WT mice,
ATGL knockout and WT mice, and saline- and nicotinic acid-treated
mice were analyzed using a 2 (genotype or nicotinic acid) × 2 (saline
or 316,243) ANOVA followed by LSD post hoc analysis, as
appropriate. In some instances, data were log transformed to ensure
normal distribution. Significance was set at P < 0.05 and data are
shown as means ± SE.

RESULTS

CL 316,243 Lowers Blood Glucose

To characterize the effects of CL in our hands, we assessed
changes in circulating metabolites and hormones 2 h following
an ip bolus injection of CL (1.0 mg/kg body wt) in fed mice. As
expected, CL treatment led to ~3- to 4-fold increases in
plasma fatty acid (Fig. 1A) and glycerol (Fig. 1B) levels.
Consistent with previous work (9), blood glucose levels (Fig.
1C) were significantly reduced in mice treated with CL, and
this was associated with an ~2.5-fold increase in plasma
insulin levels (Fig. 1D). The reductions in blood glucose and
increases in plasma insulin coincided with the induction of
IL-6 in epididymal adipose tissue (Fig. 1E), and this was
parallelized by large increases in circulating IL-6 (Fig. 1F).

IL-6 Is Not Required for CL-Induced Reductions in Blood
Glucose

Given the increases in circulating IL-6 that occurred follow-
ing CL treatment, we wanted to ascertain the role of this
cytokine in mediating the glucose lowering effects of CL. To
address this question, WT or whole body IL-6-deficient mice
were injected with CL, and alterations in circulating metab-
olites and hormones were studied. As before, plasma IL-6
levels were increased 2 h following CL treatment in WT
mice but were not detectable in either saline- or CL-treated
IL-6-deficient mice (Fig. 2A). CL-mediated increases in
plasma fatty acids (Fig. 2B) and glycerol levels (Fig. 2C) were
similar between WT and IL-6−/− mice. Likewise, blood glu-
cose levels decreased to a similar extent in both genotypes at
all time points following CL treatment (Fig. 2D). CL-mediated
increases in plasma insulin levels were similar between WT
and IL-6−/− mice (Fig. 2E).

To confirm that changes in blood glucose are independent of
plasma IL-6, we assessed metabolites and hormones 15 min
following the injection of CL, the earliest time point at which
we detected alterations in blood glucose levels (data not
shown). As seen in Fig. 3, fatty acids (A), and insulin levels (B) were increased, whereas blood glucose levels (C) were reduced ∼30% in mice treated with CL. This occurred in the absence of changes in plasma IL-6 concentrations (D).

Evidence Linking Plasma NEFAs to Reductions in Blood Glucose by CL

To assess a role of increases in fatty acids being involved in the glucose lowering effects of CL, we treated mice with nicotinic acid (250 mg/kg body wt) 15 min prior to injecting them with CL. Nicotinic acid binds to the mouse orphan G protein-coupled receptor PUMA-G (protein upregulated in macrophages by interferon-γ), and its human ortholog HM74 (13). These receptors, referred to as GPR109, are more highly expressed in adipose tissue than in other tissues, such as skeletal muscle, liver, and pancreas (27), that could be involved in the glucose lowering effects of CL. Activation of GPR109 leads to reductions in cAMP and lipolysis (13), and nicotinic acid has previously been shown to reduce plasma fatty acids and glucose-stimulated insulin secretion in fasted rats (29), CL-mediated increases in plasma fatty acid levels in mice (16) and resting and exercise-induced increases in plasma fatty acids in humans (11, 34, 35). As shown in Fig. 4A, pretreatment with nicotinic acid attenuated the glucose lowering effects of CL 30 min postinjection, and this was associated with increases in plasma fatty acids (Fig. 4B) and insulin

Fig. 1. CL 316,243 treatment reduces blood glucose and increases plasma insulin. Male C57BL6 mice were injected with CL 316,243 (1.0 mg/kg body wt ip) or an equivalent volume of sterile saline, and changes in plasma NEFA (A), plasma glycerol (B), blood glucose (C), plasma insulin (D), and IL-6 mRNA expression in epididymal adipose tissue (eWAT) and plasma IL-6 (E) were determined 2 h postinjection. Data are presented as means ± SE for 6–12 mice/group. *P < 0.05 vs. saline injected control.
concentrations of smaller magnitude than in the saline-treated animals (Fig. 4).

Although nicotinic acid has been reported to attenuate HSL activity in adipose tissue (34), and this likely explains the reduction in plasma fatty acids and subsequent blunting of CL-mediated reductions in blood glucose, longer treatment durations have been reported to modify gene expression in a variety of tissues (4). Although this is likely secondary to alterations in circulating fatty acids, we wanted to confirm the role of fatty acids in the CL-mediated reductions in blood glucose. To examine this question, we utilized whole body ATGL knockout (ATGLΔ/Δ) mice. ATGL mediates the breakdown of triacylglycerol to diacylglycerol (25). In adipose tissue explants from ATGLΔ/Δ mice, isoproterenol-stimulated lipolysis is almost completely abolished, and both fed and fasting plasma fatty acid levels are reduced (10). As shown in Fig. 5, CL-mediated increases in plasma NEFAs (A) and insulin (B) were absent in ATGL-deficient mice. CL treatment

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Fig. 2. CL 316,243-mediated reductions in blood glucose are intact in IL-6-deficient (IL-6 KO) mice. Male WT or IL-6 KO mice were injected with CL 316,243 (1.0 mg/kg body wt ip) or an equivalent volume of sterile saline, and changes in plasma IL-6 (A), plasma NEFA (B), plasma glycerol (C), blood glucose (D), and plasma insulin (E) were determined 2 h postinjection. Changes in blood glucose were measured 30, 60, 90, and 120 min postinjection. Data are presented as means ± SE for 6–8 mice per group. *P < 0.05 vs. saline-treated group within the same genotype. N.D., not detectable.
did not reduce, and in fact increased, blood glucose levels in ATGL knockout mice (Fig. 5C). When the effects of CL in WT mice were examined using a t-test, there was a significant (P < 0.05) reduction in blood glucose levels with treatment that was not picked up when analyzed with a 2 × 2 ANOVA. There was a strong trend (P = 0.051, 2-tailed t-test) for terminal plasma IL-6 concentrations to be higher in ATGL−/− (136.3 ± 22.8 ng/ml, n = 5) than in WT (80.3 ± 8.5 ng/ml, n = 5) mice.

**DISCUSSION**

CL has been shown to reduce blood glucose levels through a mechanism that is dependent on β3-adrenergic receptors in white adipocytes (9). In the current study, we provide several lines of evidence that suggest that increases in fatty acids are likely involved in this process. First, we measured rapid CL-mediated increases in plasma fatty acids that occurred in parallel with the earliest detectable reductions in blood glucose. Second, we discovered that attenuating CL-mediated increases in plasma fatty acids levels with nicotinic acid led to a blunted increase in insulin levels and an attenuated reduction in blood glucose. Last, we found that the ability of CL to stimulate insulin secretion and lower blood glucose was not reported (14), while in the other (20), CL did not reduce, and in fact increased, blood glucose levels in ATGL knockout mice (Fig. 5C). When the effects of CL in WT mice were examined using a t-test, there was a significant (P < 0.05) reduction in blood glucose levels with treatment that was not picked up when analyzed with a 2 × 2 ANOVA. There was a strong trend (P = 0.051, 2-tailed t-test) for terminal plasma IL-6 concentrations to be higher in ATGL−/− (136.3 ± 22.8 ng/ml, n = 5) than in WT (80.3 ± 8.5 ng/ml, n = 5) mice.

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Our data provide evidence that fatty acids mediate, to a large extent, the effects of CL on blood glucose in mice in vivo. What is not clear, however, is how an increase in fatty acids induced by CL stimulates the pancreas to secrete insulin. One potential candidate is GPR40 (G protein-coupled receptor 40), a fatty acid receptor expressed in pancreatic β-cells and insulin-secreting cell lines (12). In GPR40 knockout mice, CL-mediated increases in plasma insulin are partially attenuated (14, 20). However, in one study, the effects of CL on blood glucose were not reported (14), while in the other (20), CL treatment did not reduce blood glucose in either WT control or GPR40 knockout mice. Clearly, this is an area that requires further exploration. Regardless of the specific mechanism(s) involved, our findings show that increases in plasma fatty acids to high physiological levels are associated with increases in plasma insulin and a lowering of blood glucose levels and builds upon previous work demonstrating that fatty acids potentiate glucose-stimulated insulin release (15, 21).

While fatty acid levels have been shown to directly stimulate insulin secretion in ex vivo preparations (15, 21), there is accumulating evidence suggesting that indirect pathways could also be involved in the glucose lowering effects of CL. For
example, CL increases circulating IL-6 levels (2), and IL-6 has been shown to increase insulin secretion both directly and through a GLP-1-dependent mechanism (6). Moreover, CL-induced increases in adipose tissue IL-6 expression are prevented when fatty acid release is pharmacologically attenuated (18). Although there was a close association between increases in IL-6 and reductions in blood glucose levels following CL treatment, our data suggest that IL-6 is not a causal factor in this process. In support of this, in whole body IL-6 knockout mice, the glucose lowering effects of CL were indistinguishable.

Fig. 4. Nicotinic acid treatment attenuates CL 316,243-mediated reductions in plasma glucose. Male C57BL6 mice were treated with 250 mg/kg body wt nicotinic acid (NA) or an equivalent volume of sterile saline by ip injection 15 min prior to injections with CL 316,243 (1.0 mg/kg body wt ip), and changes in blood glucose (A), plasma insulin (B), and NEFA were determined. Blood was collected by saphenous blood draw prior to and 30 min postinjection. Data are presented as means ± SE for 5–8 mice per group. *P < 0.05 vs. pre-in the same drug group; # P < 0.05 vs. saline treated at the same time point.

Fig. 5. CL 316, 243-mediated increases in insulin and reductions in blood glucose are absent in ATGL KO mice. WT or whole body ATGL KO mice were injected ip with CL 316,243, and changes in plasma NEFA (A), insulin (B), and blood glucose (C) were determined prior to and 30 min following treatment. Data are presented as means ± SE for 5 mice per group. *P < 0.05 vs. prevalue within a given genotype; #P < 0.05 vs. WT group at the same time point. Please note that CL 316,243 significantly reduced blood glucose levels in WT mice when this was analyzed by t-test.
able from those in WT animals measured at multiple time points postinjection, and this occurred independently of differences in plasma fatty acid levels. Although it has recently been shown that fasting-induced increases in plasma fatty acids are reduced in IL-6-deficient mice (37), this could likely be attributed to reductions in circulating catecholamine levels in these mice (30), as the data from the current study and previous work from our laboratory (32) demonstrate that both in vivo and ex vivo lipolytic responsiveness is intact in IL-6 knockout mice. In addition to normal reductions in blood glucose in response to CL treatment in IL-6-deficient mice, we demonstrate a clear temporal dissociation between increases in plasma IL-6 concentrations and reductions in blood glucose levels following CL treatment. That is, reductions in blood glucose levels were observed 15 min following an injection of CL in the absence of increases in plasma IL-6. Lastly, in ATGL−/− mice, the glucose lowering effects of CL were abolished despite a strong trend for higher terminal levels of IL-6 in these mice. These lines of evidence argue against IL-6 mediating the glucose lowering effects of CL.

Previous work has shown that exercise-induced increases in IL-6 potentiate glucose-stimulated insulin release and glucose clearance (6). However, in the current study, despite an extremely robust increase in plasma IL-6 (~2-fold greater than that measured by Ellingsgaard et al. (6)), this did not appear to be required for the glucose lowering effects of CL. This could be related to the prolonged vs. transient increase in circulating IL-6 seen with CL treatment compared with exercise, or perhaps it could be attributed to differences in the site of IL-6 secretion, muscle in the case of exercise and adipose tissue most likely with CL. Alternatively, a glucose challenge may need to be superimposed on top of the elevations in IL-6 in order to observe a role for this cytokine in insulin secretion.

In summary, we have provided novel evidence demonstrating that CL-mediated increases in plasma insulin, which presumably lead to reductions in blood glucose, involve increases in plasma fatty acids, and that IL-6 does not appear to be involved in this process. Our findings provide important insight into the glucose lowering effects of CL and suggest that transient increases in fatty acids might serve as an effective approach to stimulate insulin secretion in conditions of impaired glucose homeostasis. The current data also hint at a fundamental and previously unappreciated aspect of adipose tissue and integrative physiology. CL causes large increases in adipose tissue lipolysis and plasma fatty acid concentrations that in all likelihood outstrip any increase in metabolic demand and need for fatty acids that would occur with acute CL treatment. The increase in fatty acid-mediated insulin release may thus serve as a signal to reduce circulating fatty acid levels through attenuating adipose tissue lipolysis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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AUTHOR CONTRIBUTIONS


