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Article

An Autocrine Lactate Loop Mediates Insulin-Dependent Inhibition of Lipolysis through GPR81

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SUMMARY

Lactate is an important metabolic intermediate released by skeletal muscle and other organs including the adipose tissue, which converts glucose into lactate under the influence of insulin. Here we show that lactate activates the G protein-coupled receptor GPR81, which is expressed in adipocytes and mediates antilipolytic effects through G_i-dependent inhibition of adenylyl cyclase. Using GPR81-deficient mice, we demonstrate that the receptor is not involved in the regulation of lipolysis during intensive exercise. However, insulin-induced inhibition of lipolysis and insulin-induced decrease in adipocyte cAMP levels were strongly reduced in mice lacking GPR81, although insulin-dependent release of lactate by adipocytes was comparable between wild-type and GPR81-deficient mice. Thus, lactate and its receptor GPR81 unexpectedly function in an autocrine and paracrine loop to mediate insulin-induced antilipolytic effects. These data show that lactate can directly modulate metabolic processes in a hormone-like manner, and they reveal a new mechanism underlying the antilipolytic effects of insulin.

INTRODUCTION

Lactate is produced from glucose through glycolysis and the conversion of pyruvate by lactate dehydrogenase (Meyerhof and Kiessling, 1935). It serves as a precursor for hepatic gluconeogenesis and may also be an energy substrate for aerobic oxidation via the citric acid cycle in various peripheral tissues (Brooks, 2002; Kreisberg, 1980). The role of lactate in the delivery of oxidative and gluconeogenic substrates is described by the "lactate shuttle" concept (Brooks, 2009). The skeletal muscle is regarded as the major site of lactate production. While it forms and utilizes lactate continuously under resting conditions, lactate formation increases during exercise (Bergman et al., 1999; Margaria et al., 1933). Also, the adipose tissue is an important source for lactate (Crandall et al., 1983; DiGirolamo et al., 1992; Ellmerer et al., 1998; Jansson et al., 1990; Marin et al., 1987). It can convert more than 50% of the metabolized glucose to lactate, a process stimulated by insulin and glucose uptake (Coppack et al., 1989; Hagstrom et al., 1990; Henry et al., 1996; Jansson et al., 1994; Qvisth et al., 2007).

The major role of the adipose tissue is to store energy in the form of triglycerides, which are constantly turned over by lipolysis and re-esterification (Duncan et al., 2007; Wang et al., 2008). Net lipolysis increases when energy demands are high and insulin levels are low, for example, during mild and intermediate exercise as well as during fasting, while it decreases postprandially after energy uptake. The second messenger cyclic AMP (cAMP) plays an important role in the regulation of lipolysis, as it can activate lipolytic enzymes via stimulation of the cAMP-dependent kinase (Langin, 2006; Zechner et al., 2005). During exercise and starvation, activation of β-adrenergic receptors induces lipolysis through the activation of cAMP formation by adenylyl cyclase, a process mediated by the G protein G_s (Horowitz, 2003; Ros et al., 1989). Insulin is the major hormone exerting an antilipolytic effect in the fed state. Its action is mediated by phosphatidylinositol-3-kinase (PI-3-kinase)-dependent activation of phosphodiesterase 3B (PDE3B) resulting in an increased rate of cAMP degradation (Degerman et al., 1998; Duncan et al., 2007; Langin, 2006). Several other antilipolytic regulators like adenosine, prostaglandin E_2, neuropeptide Y, 3-hydroxy-butyrate, or the antidyslipidemic drug nicotinic acid act through G_i-coupled receptors, resulting in an inhibition of cAMP formation via adenylyl cyclase (Gille et al., 2008; Granneman and Moore, 2008; Langin, 2006; Wang et al., 2008).

Here we show that lactate activates the G_i-coupled receptor GPR81, which is exclusively expressed on adipocytes, and that lactate released from adipocytes in response to glucose and insulin mediates insulin-induced antilipolysis by activation of GPR81.

RESULTS

Lactate Activates the G Protein-Coupled Receptor GPR81

GPR81 is an orphan G protein-coupled receptor (Lee et al., 2001) closely related to GPR109A, a receptor activated by the

antidyslipidemic drug nicotinic acid as well as by the ketone body 3-hydroxy-butyrate (Gille et al., 2008). Given that all known natural and synthetic ligands of GPR109A carry a carboxylic acid residue, we tested various naturally occurring carboxylic acids as potential endogenous ligands for GPR81. Among a series of short-chain carboxylic acids, we found that (S)-lactate activated GPR81 when expressed heterologously in chinese hamster ovary (CHO) cells (Figure 1A and data not shown). Structurally related substances like pyruvate, (S)-alanine, or 3-hydroxy-butyrate had no activity, and the effect of (S)-lactate was specific for GPR81, as the related receptors GPR109A and GPR109B were not activated by lactate (Figure 1A). (S)-lactate activated both human and mouse GPR81 with an EC_{50} of 1.5 mM when tested in the GTPγS binding assay and about 7 mM when tested in the Ca^{2+}-aequorin assay (Figures 1B and 1C). (S)-lactate, the metabolically relevant isomer, was much more potent than (R)-lactate (Figure 1B). In cells expressing GPR81 together with the β_2-adrenergic receptor, lactate was able to decrease intracellular cAMP levels raised by the β-adrenergic receptor agonist isoproterenol in a pertussis toxin-sensitive manner (Figure 1D). This indicates that GPR81 is coupled to G_i-type G proteins.

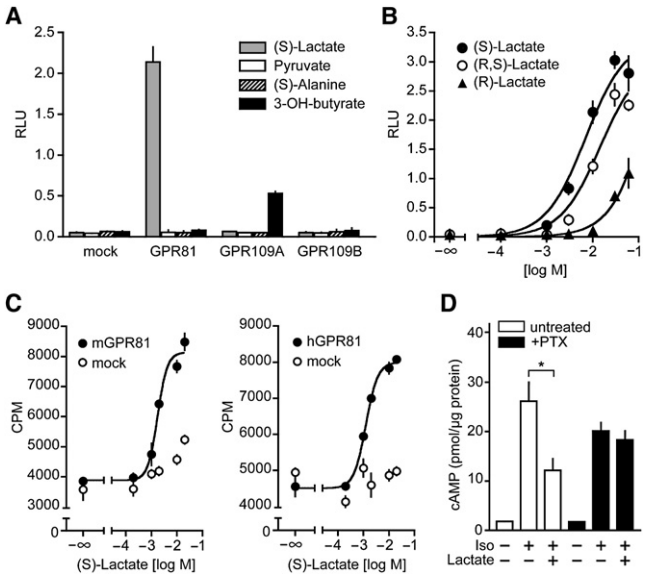

Figure 1. Identification and Characterization of GPR81 Agonists

(A) Effect of the indicated substances at a concentration of 10 mM on $[Ca^{2+}]_i$ in CHO-K1 cells expressing GPR81, GPR109A, or GPR109B together with a Ca^{2+}-sensitive bioluminescent fusion protein and the promiscuous G protein α subunit $G\alpha_{15}$. RLU, relative light units.

(B and C) Effect of increasing concentrations of the indicated lactate stereoisomers on $[Ca^{2+}]_i$ in CHO-K1 cells transfected with cDNA encoding GPR81 (B) or on the binding of GTPγS in membranes prepared from HEK293 cells transfected with an empty vector (mock) or a vector encoding human (hGPR81) or mouse GPR81 (mGPR81) (C).

(D) CHO-K1 cells cotransfected with GPR81 and the β_2-adrenergic receptor were untreated or treated overnight with 100 ng/ml pertussis toxin (PTX). Cells were then incubated in the absence or presence of 10 mM (S)-lactate and/or 1 μM isoproterenol (Iso), and cAMP levels were determined as described in the Experimental Procedures. Shown are mean values ± SEM of three independently performed experiments. *$p \leq 0.05$.

GPR81 Mediates Antilipolytic Effects of Lactate

Although it has recently been reported that lactate activates GPR81 (Cai et al., 2008; Liu et al., 2009) and that the receptor is coupled to G_i (Ge et al., 2008), its function is still unknown. Earlier reports indicated that GPR81 expression is restricted to adipocytes in mouse and human (Wise et al., 2003). To search for other potential sites of expression, we generated a bacterial artificial chromosome (BAC)-based transgenic mouse line expressing monomeric red fluorescent protein (mRFP) under the control of the mouse GPR81 promoter (for details, see the Experimental Procedures). In three independent transgenic lines we found expression indeed to be restricted to adipocytes of subcutaneous, visceral, and epididymal white adipose tissue as well as of brown adipose tissue (Figure 2A and data not shown). To further explore the physiological role of GPR81, we used GPR81-deficient mice (for details, see the Experimental Procedures). Mice lacking GPR81 were viable and showed no obvious abnormalities. In the past, lactate has been shown to inhibit lipolysis in various species (Bjorntorp, 1965; Boyd et al., 1974; De Pergola et al., 1989; Dieterle et al., 1969; Green and Newsholme, 1979; Miller et al., 1964). Consistent with this, we found that lactate dose-dependently induced an inhibition of lipolysis in isolated murine adipocytes from epididymal fat pads in a pertussis toxin-sensitive manner (Figure 2B). The antilipolytic effect of lactate in vitro was not seen in GPR81-deficient adipocytes (Figure 2C). While pretreatment of adipocytes with pertussis toxin also blocked the antilipolytic effect of the adenosine A_1 receptor agonist (R)-N^6-(2-phenylisopropyl)-adenosine (PIA), PIA was still able to inhibit lipolysis in GPR81-deficient adipocytes (Figures 2B and 2C), indicating that lack of GPR81 did not affect responsiveness of adipocytes to antilipolytic stimuli acting via G protein-coupled receptors in general.

We then injected lactate i.p. in doses sufficient to reach a plasma concentration of about 15 mM. The increase in the lactate plasma concentration was accompanied by a decrease in the free fatty acid plasma concentration in wild-type mice. However, in GPR81-deficient mice a comparable elevation of the lactate plasma concentration had no effect on free fatty acid levels (Figure 2D). The ability of GPR81-deficient mice to respond to antilipolytic agents acting via G_i-coupled receptors was not affected in general, as the adenosine A_1 receptor agonist PIA decreased free fatty acid levels to comparable extents in wild-type and GPR81-deficient mice (Figure 2D). Thus, lactate in millimolar concentrations exerts an antilipolytic effect that is mediated by GPR81.

GPR81 Is Not Involved in the Regulation of Lipolysis during Intensive Exercise

Under normal conditions the plasma concentration of lactate is in the range of 0.5 and 2 mM (Huckabee, 1958; Marbach and Weil, 1967; Niessner and Beutler, 1973), a concentration which would be too low to lead to strong GPR81 activation. However, systemic lactate concentrations as well as localized concentrations of lactate in fat tissue can increase several-fold under certain conditions (DiGirolamo et al., 1992; Hagström-Toft et al., 1997; Kreisberg, 1980). The classic situation in which plasma levels of lactate are increased is intensive exercise resulting in lactate plasma levels as high as 10–15 mM (Osnes and Hermansen, 1972; Turrell and Robinson, 1942). Since there is a correlation between the increase of plasma lactate levels and

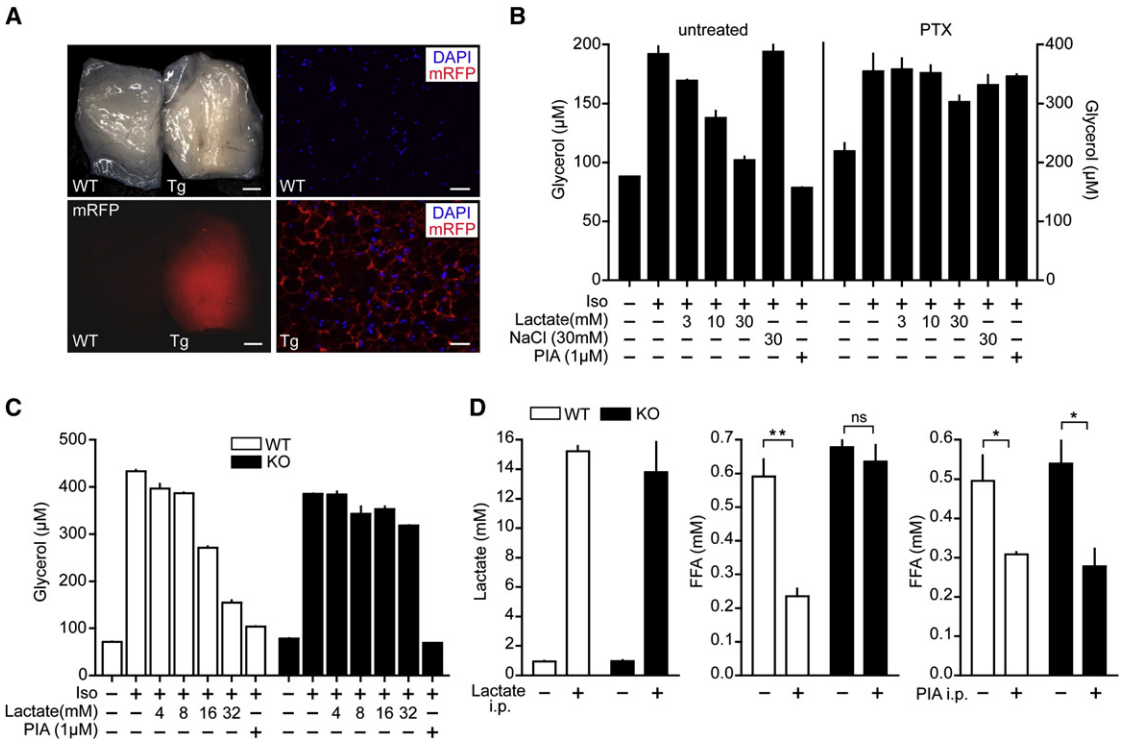

Figure 2. Expression of GPR81 and Generation of GPR81-Deficient Mice

(A) Whole-mount view and corresponding fluorescent image of subcutaneous adipose tissue (left panels) or fluorescent image of adipose tissue sections from wild-type mice (WT) or mice transgenic for the mRFP expressing GPR81 reporter construct (Tg). Scale bars, 2 mm (left) and 50 μm (right).

(B) Wild-type adipocytes were treated in the absence or presence of 200 ng/ml pertussis toxin for 5 hr. Thereafter, glycerol release was determined after incubation without or with 50 nM (−)isoproterenol (Iso) and the indicated concentrations of lactate or of the adenosine A_1 receptor agonist PIA. NaCl was tested as a control since lactate was applied as a sodium salt.

(C) Adipocytes were isolated from wild-type (WT) or GPR81-deficient mice (KO), and glycerol release was determined after incubation without or with 50 nM (−)isoproterenol (Iso) and the indicated concentrations of (S)-lactate or PIA. Shown are mean values of triplicates ± SD.

(D) Wild-type mice (WT) or GPR81-deficient mice (KO) were injected with 1.25 mg/g lactate or PIA (0.15 nmoles/g). Fifteen minutes later, lactate and free fatty acid (FFA) plasma concentrations were determined as described in the Experimental Procedures. Shown is a representative experiment with four animals per group. *$p \leq 0.05$; **$p \leq 0.01$; ns, not significant.

the decrease in fatty acid mobilization during intensive exercise, and since lactate can inhibit lipolysis, we tested whether GPR81 mediates a potential antilipolytic effect of lactate under various conditions of intensive exercise in treadmill experiments (Figure 3A). After a training period of several days (for details, see the Experimental Procedures), plasma lactate levels reached about 10 mM under exercise (see Figure 3B), a plasma concentration sufficient to exert an antilipolytic effect after i.p. injection of lactate in wild-type but not in GPR81-deficient mice (Figure 2D and see Figure S1 available online). However, there was no difference in plasma levels of glycerol and free fatty acids between exercising wild-type and GPR81-deficient mice (Figures 3C and 3D). Thus, we conclude that GPR81 does not play a critical role in regulating lipolysis during intensive exercise.

GPR81 Mediates Insulin-Dependent Antilipolysis

Since adipocytes have been shown to release lactate, a process strongly increased upon insulin-induced glucose uptake (DiGirolamo et al., 1992), we hypothesized that the locally released lactate acts in an autocrine or paracrine fashion and exerts an antilipolytic effect when plasma glucose levels are elevated. Performing microdialysis in subcutaneous adipose tissue of wild-type mice, we observed that i.p. injection of glucose resulted in an increase in the lactate concentration in adipose tissue while the release of free fatty acids declined (Figures 4A and 4B). Interestingly, in GPR81-deficient mice, a comparable increase in the lactate concentration could be seen while the decrease in lipolysis was strongly reduced (Figures 4A and 4B), although insulin plasma levels were comparable between wild-type and GPR81-deficient mice (Figure 4C). Very similar defects in GPR81-deficient mice were observed on a systemic level. An i.p. glucose challenge of fasted wild-type and GPR81-deficient mice resulted in increased systemic levels of glucose, lactate, and insulin in both groups (Figures 4D–4G and Figure S2). However, the decrease in systemic free fatty acid and glycerol levels seen in wild-type mice was strongly reduced in GPR81-deficient mice (Figures 4D and 4E). Similarly, the inhibition of lipolysis seen 30 min after refeeding of fasted wild-type mice was reduced in the absence of GPR81 (Figures 4H–4K).

To test whether a role of lactate and GPR81 in insulin-induced inhibition of lipolysis can also be seen in isolated adipose tissue in vitro, we analyzed white adipose tissue from wild-type and GPR81-deficient mice (see Figures 5A and 5B). While treatment with the β-adrenergic agonist isoproterenol increased lipolysis

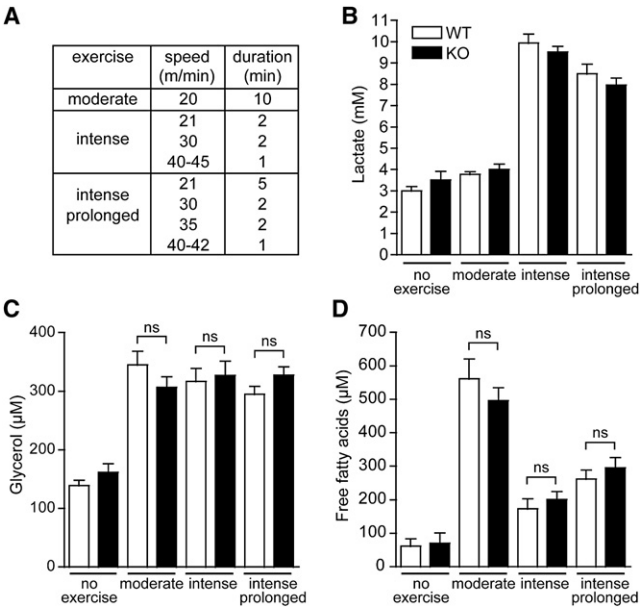

Figure 3. Effect of GPR81 on Lipolysis during Intensive Exercise

(A) Speed and duration of the different treadmill exercise programs.

(B–D) Lactate (B), glycerol (C), or free fatty acid plasma concentrations (D) were determined in wild-type (WT) or GPR81-deficient mice (KO) before (no exercise) and after the indicated treadmill exercise programs (n = 7–13 per group; ns, not significant).

Shown are mean values ± SEM.

both in wild-type and GPR81-deficient adipocytes, addition of insulin in the presence of 300 mg/dl glucose strongly inhibited lipolysis only in adipocytes from wild-type mice, whereas the antilipolytic effect was strongly reduced in adipocytes from GPR81-deficient mice (Figures 5A and 5B).

Insulin exerts its antilipolytic effects by inducing a decrease in cAMP levels, a process involving activation of the cAMP-degrading enzyme PDE3B (Degerman et al., 1997). To test whether insulin-induced decreases in cAMP levels are affected by GPR81 deficiency, we determined intracellular cAMP levels in mouse adipose tissue before and after an in vivo glucose challenge of wild-type and GPR81-deficient mice. cAMP levels strongly decreased after i.p. injection of glucose into wild-type mice. However, this effect was reduced by more than 50% in GPR81-deficient mice (Figure 5C). This indicates that lactate, which is released from adipocytes upon insulin-dependent glucose uptake, inhibits cAMP production via its receptor GPR81 and thereby mediates the postprandial inhibition of lipolysis.

The Role of GPR81 in Metabolic Regulation

We then analyzed glucose tolerance and insulin sensitivity in wild-type and GPR81-deficient animals (Figures 6A and 6B). Neither in glucose tolerance assays nor in insulin tolerance tests did we see an obvious difference between wild-type mice and mice lacking GPR81. We also followed the body weight of mice over several months. Under normal chow we did not observe any difference in body weight between GPR81-deficient and wild-type mice. However, under high-fat diet GPR81-deficient mice had a reduced weight gain compared to wild-type mice (Figure 6C). This indicates that the lactate/GPR81-dependent antilipolytic effect is involved in the weight gain under hypercaloric diet. Glucose tolerance was comparable between wild-type and GPR81-deficient mice under high-fat diet (Figure S5). The impaired antilipolytic effects of insulin observed in GPR81-deficient mice under normal chow diet were still present under high-fat diet conditions. While wild-type and GPR81-deficient animals kept under high-fat diet showed a reduced antilipolytic effect reflecting the insulin resistance of animals kept under high-fat diet, the antilipolytic effect was further reduced in mice lacking GPR81 kept on high-fat diet (Figures 6D–6G). These data clearly show that the lactate/GPR81-dependent antilipolytic mechanism is still operating in diabetic mice.

DISCUSSION

Nutrients and their metabolites provide energy and substrates for multiple metabolic processes. However, they can also exert regulatory effects by activating specific membrane receptors. Recently it has become clear that eukaryotes use membraneous G protein-coupled receptors to sense the concentration of nutrients and their metabolites in the metabolic or gustatory system (Brown et al., 2005; Chandrashekar et al., 2006; He et al., 2004; Holsbeeks et al., 2004). Here we report that the orphan G protein-coupled receptor GPR81 functions as a receptor for lactate, a central intermediate of energy metabolism. GPR81 couples to G_i-type G proteins and is expressed on adipocytes where it mediates lactate-induced antilipolytic effects. We show that insulin-dependent glucose uptake in adipocytes results in the local release of lactate, which in an autocrine fashion induces inhibition of lipolysis. In animals lacking GPR81, insulin-dependent inhibition of lipolysis as well as insulin-induced decreases in cAMP levels are almost completely abrogated. Thus, besides its well-established role as an intermediate of the energy metabolism, lactate functions as an autocrine or paracrine signaling molecule that mediates insulin-induced inhibition of adipocyte lipolysis and thereby critically contributes to the change in metabolic fluxes during the transition from the fasted to the fed state.

Especially during exercise, the skeletal muscle is the major site of lactate production in the body. During low-intensity exercise, fatty acid oxidation is the major source of energy. However, with increasing exercise intensity, the anaerobic degradation of carbohydrates via increased glycogenolysis and glycolysis to lactate prevails (Brooks and Mercier, 1994; Romijn et al., 1993). Since the accumulation of lactate in the blood during intensive exercise coincides with the decrease in fatty acid oxidation, a direct effect of lactate on fatty acid release from adipocytes has been suggested (Boyd et al., 1974; Fredholm, 1971; Issekutz and Miller, 1962). While a causal link between elevated lactate levels and a decreased fatty acid formation and oxidation is plausible, no clear proof for this concept has been provided, and the issue has remained controversial (Trudeau et al., 1999). Since GPR81 would be in an ideal position to mediate lactate-induced antilipolytic effects during exercise, we tested whether GPR81 deficiency has an effect on free fatty acid and glycerol plasma levels of exercising mice. When mice were trained to exercise resulting in lactate plasma levels, which

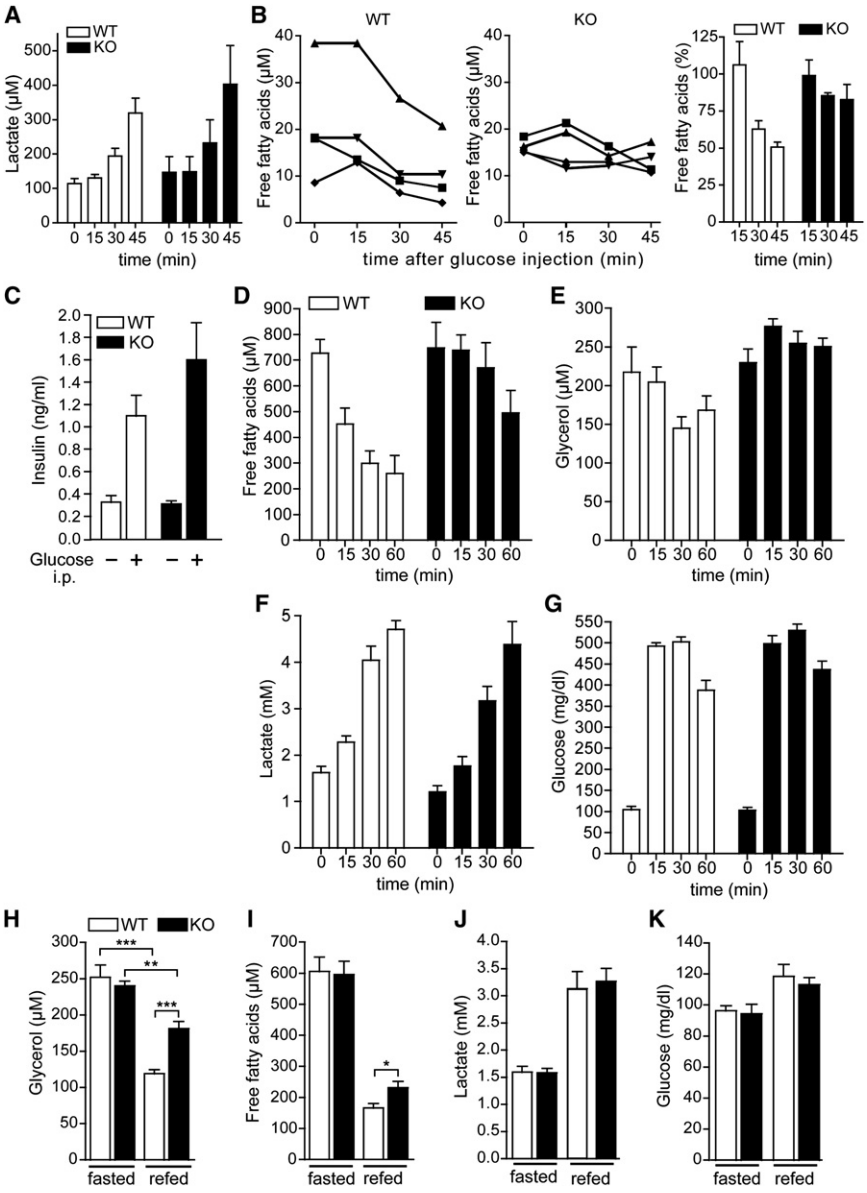

Figure 4. The Role of GPR81 in Insulin-Dependent Antilipolysis

(A–C) Wild-type (WT) and GPR81-deficient mice (KO) were implanted with a microdialysis probe in the subcutaneous adipose tissue. At time point 0′, mice were injected with 3 mg/g glucose, and lactate (A) or free fatty acid levels (B) in the dialysate were determined at the indicated time points. (B) The left and middle panels show absolute values of four independently performed experiments in wild-type (WT) and GPR81-deficient mice (KO), respectively. The right panel shows normalized values of four independently performed experiments ± SEM. Insulin plasma concentrations were determined in parallel (n = 5) (C).

(D–G) Wild-type and GPR81-deficient mice anaesthetized with isoflurane were injected i.p. with 3 mg/g glucose, and plasma free fatty acids (D), glycerol (E), lactate (F), and glucose levels (G) were determined at the indicated time points (n = 6–9 per group).

(H–K) Wild-type (WT) and GPR81-deficient mice (KO) were fasted for 12 hr during the light phase and refed for 30 min at the beginning of the dark phase. Before (fasted) and after refeeding (refed), plasma glycerol (H), free fatty acid (I), lactate (J), and glucose levels (K) were determined (n = 7 per group). *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Shown are mean values ± SEM.

are able to induce strong antilipolytic effects, free fatty acid plasma concentrations remained on comparable levels in wild-type and GPR81-deficient animals. Thus, although lactate has an antilipolytic activity under resting conditions which involves GPR81, during intensive exercise this effect may not be sufficient to overcome the strong stimulation of lipolysis via sympathetic activation (Horowitz, 2003). Alternatively, it is also conceivable that GPR81-independent mechanisms are involved in the relative reduction of the lipolytic rate during intensive exercise.

The rapid decrease in lipolytic activity in the presence of sufficient supply of carbohydrates after a meal is an important metabolic regulatory process which preserves energy stores in adipocytes. Food uptake inhibits net lipolysis primarily through the action of insulin, which by binding to its receptor on adipocytes increases glucose uptake and exerts a strong and potent antilipolytic effect that is primarily mediated by a decrease in intracellular cAMP levels (Duncan et al., 2007; Wang et al., 2008). The classic pathway linking insulin receptor activation in adipocytes with the suppression of cAMP levels involves the PI-3-kinase-dependent activation of protein kinase B/Akt, which in turn phosphorylates and activates PDE3B, the major phosphodiesterase isoform responsible for cAMP degradation in adipocytes (Degerman et al., 1998). Our data indicate that the mechanism underlying the insulin-induced drop in adipocyte cAMP levels is more complex and involves insulin-stimulated release of lactate from adipocytes. Adipocytes have been shown to be a major site of lactate production by conversion of glucose (DiGirolamo et al., 1992), a process strongly increased by insulin (Hagstrom et al., 1990; Henry et al., 1996). Lactate released from adipose tissue has been regarded primarily as a source for hepatic gluconeogenesis and glycogen synthesis. The fact that the lactate receptor GPR81 is specifically expressed on adipocytes suggested that an increased lactate release upon insulin-stimulated glucose uptake would in an autocrine fashion result in G_i-mediated inhibition of adenylyl cyclase, thereby contributing to the antilipolytic effect of insulin. Indeed, while local free fatty acid levels in the adipose tissue as well as systemic free fatty acid levels dropped after an acute glucose challenge of wild-type mice, this effect was greatly diminished in animals lacking the lactate receptor GPR81. Comparable effects could be observed in isolated wild-type and GPR81-deficient adipose tissue treated with insulin.

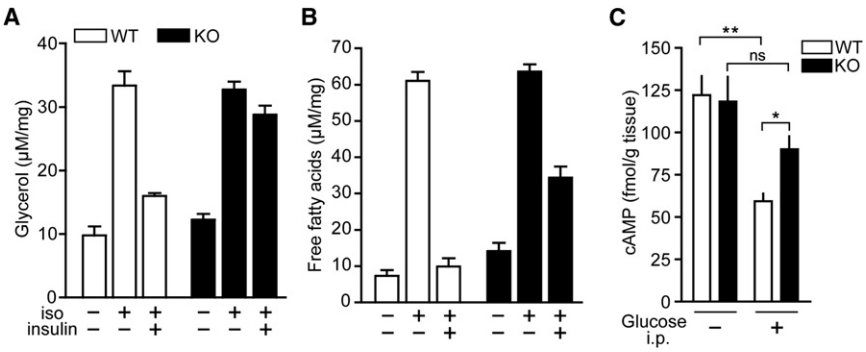

Figure 5. GPR81 Involvement in Insulin-Dependent Antilipolytic Effects and Decreases in cAMP Levels

(A and B) White adipose tissue explants from wild-type (WT) or GPR81-deficient mice (KO) were incubated without or with 10 nM (−)isoproterenol (iso) in the presence or absence of 20 ng/ml insulin for 4 hr. Thereafter, concentrations of glycerol (A) and free fatty acids (B) in the supernatant were determined. Shown are mean values ± SEM of quadruplicates of a representative experiment.

(C) Wild-type (WT) and GPR81-deficient mice (KO) were injected with 3 mg/g glucose. After 30 min, epididymal fat pads were removed, and cAMP levels were determined as described in the Experimental Procedures (n = 5 per group). Shown are mean values of triplicates ± SEM. *$p \leq 0.05$; **$p \leq 0.01$; ns, not significant.

Also, the decrease in adipocyte cAMP levels seen after an acute glucose challenge in wild-type mice was strongly reduced in mice lacking GPR81, indicating that the activation of PDE3B alone was not sufficient to mediate the effect of insulin on cAMP levels and lipolytic activity. The fact that insulin-dependent antilipolysis is strongly inhibited after blockade of PDE3B or in the absence of PDE3B in PDE3B-deficient mice (Choi et al., 2006; Eriksson et al., 1995) does not speak against the mechanism described here, since PDE3B inhibition or elimination does not only block the effect of insulin on cAMP degradation but also results in an increase in basal cAMP levels. The latter effect is unlikely to be overcome by insulin-induced inhibition of cAMP formation via lactate and GPR81. Thus, a dual regulation of adipocyte cAMP levels through the stimulation of cAMP degradation via PDE3B and the inhibition of cAMP formation via lactate and GPR81 (Figure 7) is necessary for the rapid and efficacious antilipolytic effect of insulin on adipocytes. This mechanism explains the long-known phenomenon that lactate can potentiate the antilipolytic effect of insulin (Green and Newsholme, 1979) and is the basis for a well-controlled switch of energy sources from lipids to carbohydrates upon feeding. A lack of insulin-dependent antilipolytic effects has been shown in adipocyte-specific insulin receptor-deficient mice to strongly reduce the increase in fat mass under the condition of a hypercaloric diet (Bluher et al., 2002). Consistent with this, GPR81-deficient mice had a reduced weight gain compared to wild-type mice under high-fat diet. The reduced weight gain was not accompanied by an increase in glucose tolerance compared to wild-type animals. This may be due to transient elevations in free fatty acid levels in GPR81-deficient mice, which may negatively influence insulin sensitivity.

These data also indicate that lactate which has traditionally been viewed primarily as a product of glucose metabolism and as a source for hepatic gluconeogenesis exerts by itself hormone-like effects by activating a specific G protein-coupled receptor. Together with the recently discovered G protein-coupled receptors for free fatty acids, ketone bodies, β-oxidation intermediates, or citric acid cycle intermediates (Ahmed

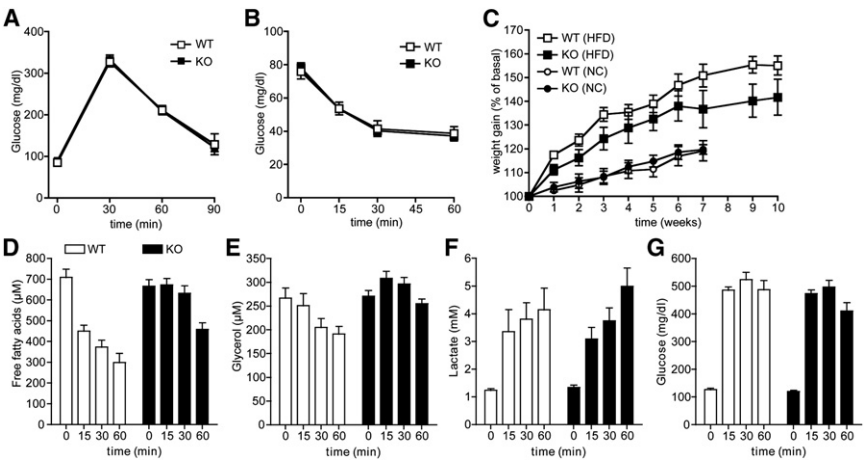

Figure 6. Role of GPR81 in Metabolic Regulation

(A) Wild-type (WT) or GPR81-deficient mice (KO) were fasted overnight and injected i.p. with 3 mg/g glucose. Thereafter, plasma glucose levels were determined at the indicated time points (n = 10 per group).

(B) Wild-type (WT) or GPR81-deficient mice (KO) were fasted overnight and injected i.p. with 0.75 mU/g insulin, and plasma glucose levels were determined at the indicated time points (n = 8 per group). Shown are mean values ± SEM.

(C) Wild-type (WT) or GPR81-deficient mice (KO) were fed a high-fat diet (HFD) or normal chow (NC). The gain in body weight was expressed as percentage of initial body weight (n = 6–10 per group). Shown are mean values ± SEM.

(D–G) Overnight fasted wild-type (WT) and GPR81-deficient mice (KO) anaesthetized with isoflurane were injected i.p. with 3 mg/g glucose, and plasma levels of free fatty acids (D), glycerol (E), lactate (F), and glucose (G) were determined at the indicated time points (n = 10 per group). Shown are mean values ± SEM.

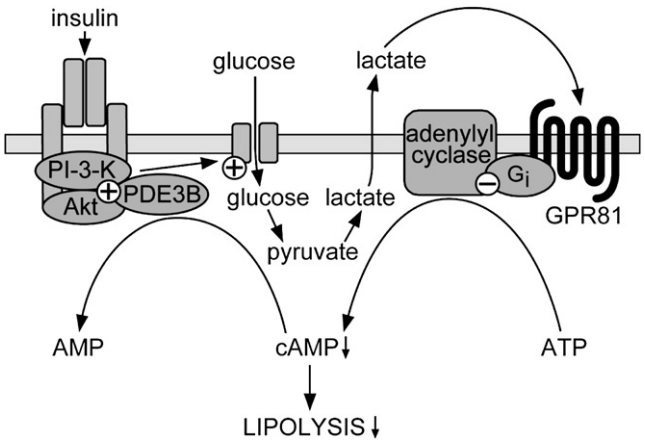

Figure 7. Model of the Mechanisms Underlying Insulin-Induced Inhibition of Adipocyte Lipolysis via PDE3B-Mediated cAMP Degradation and Lactate/GPR81-Dependent Inhibition of cAMP Formation
For details, see text. PI-3-K, phosphatidylinositol-3-kinase; PDE3B, phosphodiesterase 3B.

et al., 2009; Brown et al., 2005; He et al., 2004; Taggart et al., 2005), the lactate receptor GPR81 forms a group of metabolic sensors, which detect local and systemic concentrations of key metabolites in order to adjust metabolic fluxes to varying metabolic states. Within this general concept, adipocytes use GPR81 to indirectly sense the availability of sufficient amounts of glucose, since high glucose levels result in increased insulin-dependent glucose uptake and subsequent conversion of glucose to lactate, which then activates GPR81. Activation of GPR81 decreases the net rate of lipolysis in order to save energy stored in adipocytes when glucose is available as an energy source. Alterations in GPR81 expression or function may contribute to dysfunctions of the metabolic system.

EXPERIMENTAL PROCEDURES

Materials

(−)-Isoproterenol hydrochloride; PIA ([R]-N^6-[2-phenylisopropyl]-adenosine); sodium (S)-, (R)-, and (R,S)-lactate; sodium pyruvate; (S)-alanine; sodium (R,S)-3-hydroxybutyrate and bovine serum albumin (free fatty acid-free); and collagenase type II (474 U/mg) were from Sigma. Pertussis toxin was obtained from Calbiochem. Adenosine deaminase was obtained from Merck.

Cell Transfection and Determination of $[Ca^{2+}]_i$

CHO-K1 cells stably transfected with a Ca^{2+}-sensitive bioluminescent fusion protein consisting of aequorin and GFP (Baubet et al., 2000) were seeded in 96-well plates and were transfected with the indicated cDNAs (60 ng/well) encoding receptors and the promiscuous G protein α subunit $G\alpha_{15}$ (Offermanns and Simon, 1995) using FuGENE6 reagent (Roche). Determination of $[Ca^{2+}]_i$ was performed using a luminometer plate reader (Luminoskan ascent, Lab Systems) as described (Tunaru et al., 2003, 2005).

GTPγS Binding

To directly determine GPR81-mediated G protein activation, plasma membranes were prepared from HEK293T cells transfected with cDNAs encoding the indicated receptors, and the binding of [^{35}S]GTPγS was measured in the absence or presence of the indicated ligands. Briefly, 50 μg of membrane protein was incubated for 60 min at 35°C in a total volume of 100 μl buffer containing 100,000 cpm (0.4 nM) of [^{35}S]GTPγS, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 100 mM NaCl, 10 μM GDP, and 50 mM Tris-HCl (pH 7.4). Incubation was terminated by filtration over GF-B glass fiber filters (Whatman) followed by two washes with 4 ml ice cold buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.02% CHAPS. GTPγS bound to the filters was measured using a liquid scintillation counter (Beckman).

Determination of cAMP Levels

The cAMP levels in cells transfected with GPR81 were determined as described (Tunaru et al., 2003, 2005). To analyze cAMP levels in adipose tissue, mice were anaesthetized with pentobarbital (60 mg/kg), and epididymal adipose tissue samples were taken and snap frozen in liquid nitrogen. Samples were weighed and homogenized using a rotor-stator system in ice-cold 0.1 N HCl. After centrifugation (1500 × g, 10 min, 4°C), the infranatant was carefully removed with a needle and a syringe. After centrifugation (20,000 × g, 5 min, 4°C), a 1:2 dilution of the sample was prepared for measuring cAMP using an ELISA kit following the manufacturer's instructions (Cayman).

Preparation of Adipocytes and Determination of Lipolysis

Adipocytes from mouse epididymal fat were isolated according to a modified method described by Rodbell (Rodbell, 1964). Briefly, adipocytes were digested with collagenase II (0.25 mg/ml) in a buffer containing 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 mM KH_2PO_4, 2% BSA, 4 mM glucose, and 25 mM Tris (pH 7.4) for 1 hr at 37°C. Cells were filtered through a nylon mesh and washed three times with the same buffer. Adenosine deaminase was present at 0.5 U/ml. Isolated adipocytes were incubated in the presence or absence of the indicated ligands and/or isoproterenol at 37°C in a shaking waterbath. After 2 hr samples were taken, and glycerol release from isolated adipocytes was measured using a colorimetric assay kit (Randox).

Generation of GPR81-Deficient Mice

Mice lacking GPR81 were obtained from Texas Institute of Genomic Medicine (Houston, TX). The exon encoding murine GPR81 was replaced by a cassette encoding β-galactosidase as well as neomycin resistance by homologous recombination in embryonic stem cells (ESCs). Correct targeting was verified in ESCs by Southern blotting and PCR (Figure S3). Chimeric mice were generated and bred with C57Bl/6N to produce F1 heterozygotes. Germline transmission was verified by PCR analysis. All procedures of animal care and use in this study were approved by the local animal ethics committee (Regierungspräsidium Karlsruhe, Germany).

Generation of a Transgenic GPR81 Expression Reporter

To generate GPR81 expression reporter mouse lines, a cassette consisting of the mRFP followed by a polyadenylation signal from bovine growth hormone and a module containing the β-lactamase gene flanked by frt sites was introduced into the coding ATG of the mouse GPR81 gene carried by a BAC using ET recombination as described (Wirth et al., 2008) (Figure S4). Correct recombinants were verified by Southern blotting. After FLPe-mediated recombination, the recombined BAC was injected into pronuclei of FvB/N oocytes. Transgenic offspring were analyzed for BAC insertion by genomic PCR. Three different founders were used to generate GPR81 reporter lines in which mRFP expression was determined by fluorescence microscopy of whole organs or 10–14 μm cryosections of various tissues.

Microdialysis Studies

For microdialysis studies, mice were kept under isoflurane/N_2O anesthesia, and a microdialysis probe (CMA20 Elite, 4 mm membrane length, CMA) was implanted with an introducer needle into the inguinal subcutaneous tissue. The system was connected to a perfusion pump (CMA 402, CMA) and rinsed with a running buffer consisting of 0.9% NaCl and 2% free fatty acid-free BSA for 15 min. The flow rate was set to 0.7 μl/min, and after a 10 min equilibration period fractions were collected every 15 min with a microfraction collector (CMA 142, CMA) to obtain a sample volume of 10.5 μl. One minute before the end of the second fraction period, 3 mg/g glucose was injected intraperitoneally, and the following three fractions were collected. Recovery rates for lactate and palmitate were determined under the same conditions in vitro (see Figure S6).

Determination of Insulin, Lactate, Glycerol, Glucose, and Free Fatty Acid Levels

All metabolites were measured using enzyme-based colorimetric assay kits according to the manufacturer's instructions (Randox). Insulin levels were determined using an ELISA kit (Millipore).

Treadmill Experiments

Exercise experiments were performed on a specialized animal treadmill (Columbus Instruments). On the first day, mice were allowed to get used to the device followed by a daily training period at different speeds (7 days, twice per day, 18–30 m/min for approximately 10 min). The slope of the belt was set to 5°. On the day of the experiment, mice ran according to the protocol shown in Figure 3A. Following exercise, blood samples were taken under isoflurane anesthesia.

In Vitro Lipolysis Assay

White adipose tissue explants of approximately 20 mg were incubated in a buffer containing 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 mM KH_2PO_4, 2% (w/v) BSA, 4 mM glucose, and 25 mM Tris (pH 7.4) for 4 hr at 37°C in a shaking water bath, and medium concentrations of free fatty acids and glycerol were measured. Isoproterenol was used at 10 nM, and insulin was added at 20 ng/ml. Adenosine deaminase was present at 0.5 U/ml.

In Vivo Lipolysis Assay

For determination of glucose-induced changes in lipolysis, mice were overnight fasted and injected with 3 mg/g glucose, and blood samples were taken at the indicated time points under isoflurane anesthesia. Determination of free fatty acids, glycerol, lactate, or glucose was performed as described.

Glucose Tolerance and Insulin Tolerance Tests

Glucose-tolerance tests (GTTs) in awake mice kept on high-fat diet were performed after overnight fasting followed by injection of glucose at 1 mg/g. Blood samples were drawn from the tail tip at the indicated time points. Glucose was measured using AccuCheck Glucometer (Roche). For GTT in lean mice fed with normal chow under pentobarbital anesthesia, the animals were overnight fasted and injected with 3 mg/g glucose, and blood samples were taken from the retroorbital plexus. For insulin tolerance tests, mice were injected with insulin (0.75 mU/g), and glucose levels were determined at the indicated time points.

Statistics

Statistical significance was assessed by unpaired Student's t test using GraphPad Prism software. Differences were considered statistically significant at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at doi:10.1016/j.cmet.2010.02.012.

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