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Lactate Inhibits Lipolysis in Fat Cells through Activation of an Orphan G-protein-coupled Receptor, GPR81*[S]

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Lactic acid is a well known metabolic by-product of intense exercise, particularly under anaerobic conditions. Lactate is also a key source of energy and an important metabolic substrate, and it has also been hypothesized to be a signaling molecule directing metabolic activity. Here we show that GPR81, an orphan G-protein-coupled receptor highly expressed in fat, is in fact a sensor for lactate. Lactate activates GPR81 in its physiological concentration range of 1–20 mM and suppresses lipolysis in mouse, rat, and human adipocytes as well as in differentiated 3T3-L1 cells. Adipocytes from *GPR81*-deficient mice lack an antilipolytic response to lactate but are responsive to other antilipolytic agents. Lactate specifically induces internalization of GPR81 after receptor activation. Site-directed mutagenesis of GPR81 coupled with homology modeling demonstrates that classically conserved key residues in the transmembrane binding domains are responsible for interacting with lactate. Our results indicate that lactate suppresses lipolysis in adipose tissue through a direct activation of GPR81. GPR81 may thus be an attractive target for the treatment of dyslipidemia and other metabolic disorders.

GPR81 (1) is an orphan G-protein-coupled receptor that is highly homologous to GPR109a and GPR109b. GPR109a and GPR109b were recently identified as receptors for niacin (also known as nicotinic acid) (2, 3) and subsequently characterized as receptors for the endogenous ketone body β-hydroxybutyrate (4). Niacin has been used clinically for a half-century as an effective treatment for dyslipidemia (5); however, its utility is somewhat hampered by a target-related effect on dendritic Langerhans cells, which release prostaglandin D2 in response to GPR109a stimulation, resulting in a cutaneous flushing response (6–8). GPR81 is highly expressed in fat, similar to GPR109a, but is not expressed significantly in spleen; nor is it highly detected in any other tissue, and it has thus been hypothesized to be a potential target for the treatment of dyslipidemia that would be analogous to GPR109a/niacin but without the potential side effects (9).

In this report, we demonstrate the initial identification of the ligand activity for GPR81 from the rat tissue extracts, the purification of L-lactate from porcine brain as the source of the ligand activity, and the pharmacological characterization of L-lactate as a ligand for GPR81. In addition, we show that in its physiological concentration range, L-lactate effectively inhibits lipolysis in adipocytes from humans, mice, and rats. Adipocytes from *GPR81*-deficient mice lack responses to L-lactate, indicating that the antilipolytic effect of L-lactate is mediated by GPR81. Despite a long history of being considered as waste or a by-product of metabolism, L-lactate has maintained some attention as a potential signaling molecule (10). As early as the 1960s, researchers have demonstrated significant effects of lactate on adipocytes (11); however, the mechanism by which this occurs has remained unknown. Our finding in this report provides a molecular basis for the ability of lactate to modulate lipolysis in adipocytes and establishes a new target opportunity for the treatment of dyslipidemia.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals tested as ligands for GPR81 were purchased from Sigma.

Identification of GPR81 Ligand Activity from Rat Tissues—Different rat tissues (5 g/tissue) were homogenized in cold (−30 °C) 80% ethanol at a tissue/solvent ratio of 1:8. The extracts were centrifuged at 10,000 × *g* for 30 min. The supernatants were collected, and volumes were reduced using a rotary evaporator at 30 °C. The remaining solution was centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was then passed through a C-18 Sep-pack column (Bond Elut; Varian), and the flow-through was collected and dried in a lyophilizer. The dried sample was reextracted with pure ethanol, and the supernatant was dried in a rotary evaporator, reconstituted in water, and tested for activation of GTPγS[4] incorporation, as described previously (12), in cell membranes expressing the recombinant human GPR81.

Purification of GPR81 Ligand from Porcine Brain—To purify the GPR81 ligand from porcine brain, 200 g of frozen porcine brain (Pel-Freez Biologicals) were homogenized under similar conditions as the rat tissues. The extract was centrifuged, the

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3 and Figs. 1–6.

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[4] The abbreviations used are: GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; PTX, pertussis toxin; PBS, phosphate-buffered saline; FFA, free fatty acid; ELISA, enzyme-linked immunosorbent assay; GPCR, G protein-coupled receptor; DCA, dichloroacetate; RT, reverse transcription; GHB, γ-hydroxybutyric acid; CHO, Chinese hamster ovary.

supernatant was collected, and the volume was reduced on a Rotovap at 30 °C to about 200 ml. The sample was centrifuged at 10,000 × *g* for 30 min, and the supernatant was loaded onto a C-18 Sep-pack column from Varian. The flow-through was dried in a lyophilizer and dissolved in 2 ml of distilled water. The sample was adjusted to pH 3 with concentrated HCl before loading to a Restek AllureOA column (300 × 10 mm, 5 μm, 60 Å). Preparative HPLC was run on a Waters Alliance 2790 system (flow rate 4 ml/min; mobile phases: 1 mM HCl in water (A) and acetonitrile (B); gradient: 0–10 min, 100% A). Fractions were collected and neutralized with NaOH before being tested in a GTPγS binding assay to identify the active fraction. The active fraction was lyophilized and dissolved in 0.5 ml of D_2O. The pH of the sample was adjusted to 8 with NaOH, and NMR data were acquired on a Bruker DRX600 spectrometer at 40 °C (^1H, ^{13}C APT, COSY, HSQC). NMR data were also obtained for a sample containing 15 mg of pure (L-)sodium lactate (purchased from Sigma) under the same conditions.

Molecular Cloning and Recombinant Expression of GPR81 from Different Species—GPR81 genes from humans, mice, rats, dogs, pigs, cows, and monkeys were PCR-amplified using primers listed in supplemental Table 1 and respective genomic DNAs as the templates. The PCR products were then cloned in a mammalian expression vector pCIneo (Promega), and the insert regions were sequenced to confirm the sequence identities. The expression vectors were either transiently expressed in CHO-K1 cells or stably expressed in SK-N-MC/CRE-β-gal cells as described (13).

Measuring L-Lactate from Cell Culture Media and Tissue Extracts—3T3-L1 cells were differentiated in 24-well tissue culture plates for 15 days. The adipocytes were washed using lipolysis washing buffer (Zen-Bio, Inc.) and replaced with 500 μl of lipolysis assay buffer (Zen-Bio) plus additional glucose (25 mM) with or without 5 μM recombinant human insulin (Sigma). 3T3-L1 adipocytes were then incubated in a 37 °C cell culture incubator for 3 h, and lactate production was determined using a lactate assay kit (Eton Bioscience, San Diego, CA). To measure lactate contents in tissues, different rat tissues were extracted with cold 80% ethanol (tissue/solvent ratio 1:8) and centrifuged at 10,000 × *g* at 4 °C for 30 min. The supernatants were collected and diluted with water at different dilutions. The lactate contents were then assayed using the lactate assay kit (Eton Bioscience).

Pharmacological Characterization of L-Lactate as the Ligand for GPR81—To characterize agonists for GPR81, CHO cells transiently expressing GPR81 from different species were tested using different compounds as agonists in a GTPγS binding assay. For cAMP accumulation studies, SK-N-MC/CRE-β-gal cells stably expressing human GPR81 were treated with various concentrations of L-lactate and then stimulated with forskolin. cAMP accumulation was measured as previously described (12). For pertussis toxin (PTX) treatment, cells transfected with GPR81 were treated with PTX (100 ng/ml) overnight before assaying the receptor activity.

Receptor Internalization Studies—A V5 N-terminally tagged human GPR81 expression construct was constructed by adding a V5 tag (MGKPIPNPLLGLDST) coding region at the 5′ end of the human GPR81 coding region. The DNA construct was sequenced to confirm the sequence identity. The DNA construct was transfected into CHO cells. One day after transfection, the cells were cultured in low glucose medium (50% minimum essential medium Eagle plus 50% PBS and 1% bovine serum albumin) for 3 h and then incubated with anti-V5 antibody (Invitrogen) at a concentration of 2 μg/ml diluted in the low glucose medium (described above) for 20 min in a tissue culture incubator. To detect cell surface staining of V5-GPR81 on live cells, cells were washed 3 times and then incubated with Cy3-labeled goat anti-mouse IgG. To study lactate-induced GPR81 internalization, following the incubation with anti-V5 antibody, L-lactate (final concentration 25 mM) was added to stimulate the receptor internalization for 30 min. The cells were then either digested by trypsin (0.05% diluted in low glucose medium without BSA) or not for 5 min to remove the cell surface anti-V5 antibody and washed three times with PBS. Finally, the cells were fixed with paraformaldehyde and permeabilized with Triton X-100, and internalized anti-V5 antibody was visualized by staining with a Cy3-labeled goat anti-mouse IgG antibody and viewed under a fluorescent microscope.

Western Blot Analysis of Erk Phosphorylation—SK-N-MC/CRE-β-gal cells stably expressing human GPR81 were treated with or without PTX (100 ng/ml) overnight. The cells were then treated either with or with out L-lactate (10 mM) for 5 min, and cell lysates were subjected to Western blot analysis using anti-phosphorylated Erk antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to detect phosphorylated Erk levels. The membrane was then stripped and reblotted with anti-Erk antibody (Santa Cruz Biotechnology) for total Erk levels. SK-N-MC/CRE-β-gal cells without GPR81 were used as control.

Mutagenesis Studies—Human GPR81 with a FLAG tag at the C terminus was used as the template for mutagenesis by a standard protocol. The mutant receptors were then recombinantly expressed and assayed for their responses to L-lactate in a GTPγS binding assay. All of the mutant GPR81 protein expression was verified by anti-FLAG staining.

Generation of GPR81 Knock-out Mouse—GPR81-deficient mice were generated by Deltagen (San Mateo, CA). The transmembrane domain 2 of mouse GPR81 coding region (100 bp) is replaced by a 7-kb IRES-*lacZ-neo* cassette.

Adipocyte Lipolysis Studies—3T3-L1 preadipocytes were grown in 24-well tissue culture plates and differentiated as described previously (14) for 15 days. Human subcutaneous adipocytes grown and differentiated *in vitro* in 24-well tissue culture plates were purchased from Zen-Bio. Differentiated 3T3-L1 and human primary adipocytes were washed with lipolysis washing buffer (Zen-Bio) and incubated in lipolysis assay buffer (500 μl/well; Zen-Bio) at 37 °C in a tissue culture incubator. Three hours (3T3-L1 adipocytes) or 5 h (human primary adipocytes) after incubation, glycerol and free fatty acid (FFA) content in the assay buffer were determined using a free glycerol reagent (Sigma) or a fatty acid kit (Zen-Bio). To study lipolysis in the primary mature adipocytes, subcutaneous or epididymal fat tissues were dissected from Sprague-Dawley rats or mice with 129SvJ/C57Bl/6J background. The mature adipocytes were isolated, and lipolysis studies were performed as described previously (9). Samples were taken hourly, and glycerol production and fatty acid release were determined using a

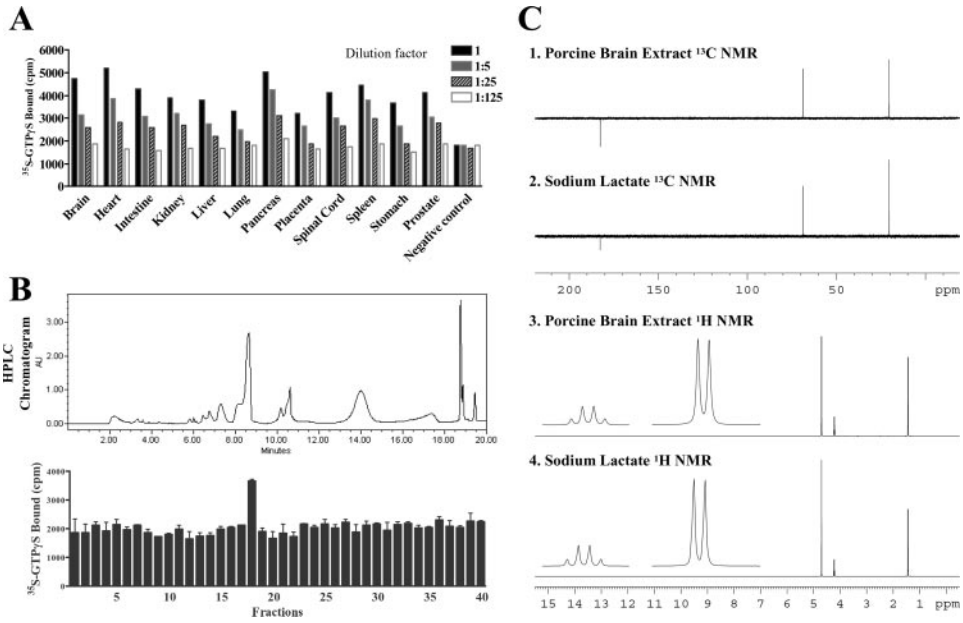

FIGURE 1. **Identification of L-lactate as a ligand for GPR81.** *A*, rat tissue extracts at various dilutions were tested for ligand activity in a GTPγS binding assay for GPR81. Extract with solvents but without tissue was used as negative control. *B*, porcine brain extract was run though HPLC using a Restek AllureOA column, and fractions were tested for GPR81 ligand activity in a GTPγS binding assay. The assay was performed in triplicates, and means ± S.E. are shown. *C*, the purified porcine brain fraction with GPR81 ligand activity was analyzed in an NMR spectrometer. ^{13}C (*1*) and ^{1}H (*2*) NMR data of the ligand were obtained, which showed identical spectra to those from pure commercial L-lactate (*3* and *4*).

free glycerol reagent (Sigma) or a fatty acid measuring kit (Zen-Bio). For human and rat adipocytes, isoproterenol was added to a final concentration of 0.5 μM to all samples to stimulate lipolysis. For 3T3-L1 adipocytes and mature adipocytes isolated from mice, lipolysis studies were performed without isoproterenol.

Quantitative RT-PCR Analysis of mRNA Expression—PCR primers for the indicated genes (supplemental Table 2) were used to analyze specified mRNA expression using a method described previously (15). cDNAs for human, rat, and mouse tissues that were used for *GPR81* mRNA quantification were purchased from Clontech (Palo Alto, CA). cDNAs from differentiated or undifferentiated 3T3-L1 cells were made in house by a standard protocol.

Detection of GPR81 Protein Expression in Mouse Tissues or Cells—A chicken antibody against mouse GPR81 (antibody MA) made against a C-terminal region of the mouse GPR81 (amino acid sequence DGANRSQRPSDGQW) was coated on an ELISA plate at a concentration of 1 μg/ml. The plate was blocked with blocking buffer (phosphate-buffered saline solution plus 0.1% Tween 20 (PBST) and 3% nonfat dry milk). Crude plasma membrane was prepared from different tissues from either wild type or GPR81-deficient mice or 3T3-L1 cells and solubilized with lysis buffer (50 mM Tris-HCl, 100 mM NaCl plus 1% Triton X-100). The lysates were centrifuged at 2000 × *g* at 4 °C for 5 min, and the clear supernatants were aliquoted into an MA antibody-coated ELISA plate in triplicates. The plate was then incubated at 4 °C overnight with shaking on a platform. The plate was washed with PBST three times and then incubated with a rabbit anti-mouse GPR81 antibody (antibody MB) against a different region of the C terminus of mouse GPR81 (amino acid sequence SLKPKRPGRTKTRRSEEMPISNLC), which was diluted into the blocking buffer at a final concentration of 1 μg/ml. The plates were incubated at room temperature for 2 h and washed with PBST followed by the incubation with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG) diluted (at a final concentration of 100 ng/ml) in blocking buffer for 2 h at room temperature. The plate was again washed with PBST and developed as described in standard ELISA protocols. Series dilutions of cell lysates from COS-7 cells expressing recombinant mouse GPR81 were included in the assay as the standards for the quantification of the relative expression levels of GPR81 from different tissues or cells. The final relative expression levels of GPR81 from different tissues were normalized by the tissue weights or the cells pellet weights for 3T3-L1 cells.

Molecular Modeling—The primary sequence alignment between bovine rhodopsin 1HZX (Protein Data Bank code) and GPR81 was determined using the program ClustalW (16). The helical alignment was further examined and refined based on the multiple sequence alignment of family A GPCRs, as described elsewhere (17). The rhodopsin structure (1HZX) was used as a template, and based on the sequence alignment, the appropriate residues of the helices of the rhodopsin were changed to the corresponding amino acid of the GPR81 using the Insight I Homology tools (distributed by Accelrys Software Inc.). The amino acid side chains were energy-minimized and placed in a reasonable conformation. For this study, the loops were discarded, and only the transmembrane bundle was used to describe the putative small molecule-binding site. The final structure was minimized with a limited cycle using the Accelrys Discover software and CVFF force field. All of the nonbonded heavy atom clashes were removed by energy minimization of the final structure. A manual docking of the ligand was followed by further energy minimization of the complex.

RESULTS

Identification and Purification of L-Lactate as a Ligand for GPR81—As an effort to identify the endogenous ligand(s) for orphan GPCR GPR81, we tested extracts from different rat tissues for ligand activity in *GPR81*-transfected cells. Surprisingly, the results showed that all tissue extracts produced apparent activity to stimulate [^{35}S]GTPγS binding in GPR81-expressing cell membranes (Fig. 1*A*) but not in control cells (not shown), with the highest activities observed in tissue extracts of heart, pancreas, and brain. To purify the ligand for GPR81, porcine brain was extracted to provide substantially more material than was accessible via rat brain tissue. The majority of the porcine

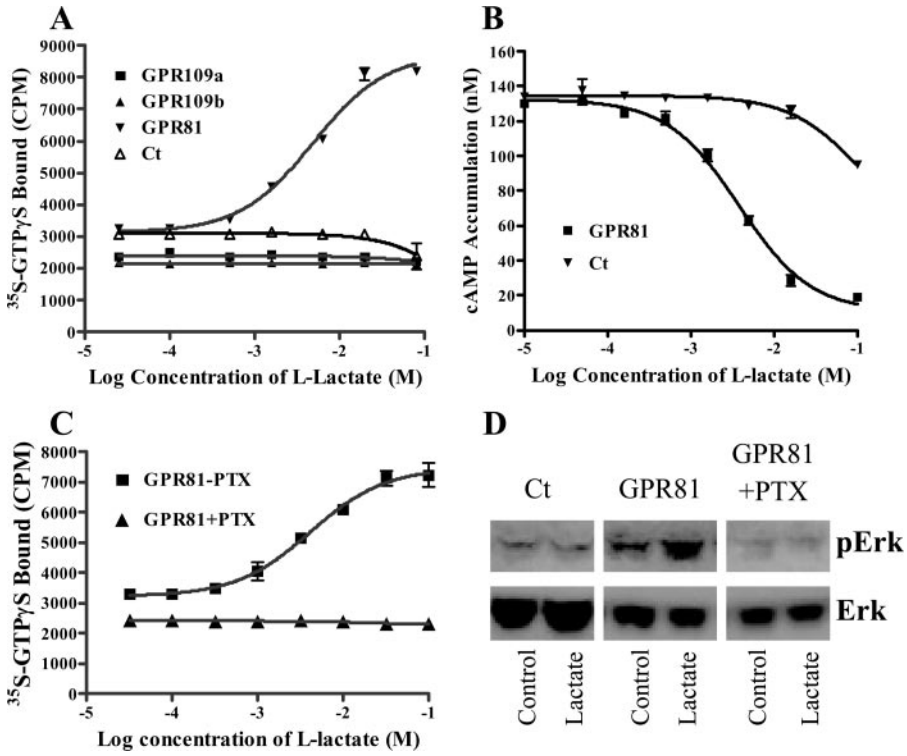

FIGURE 2. **Pharmacological characterization of GPR81.** *A*, L-lactate stimulates [^{35}S]GTPγS incorporation in GPR81 expression cells but not the control cells (*Ct*) or cells expressing niacin receptors GPR109a or GPR109b. *B*, L-lactate inhibits cAMP accumulation in GPR81-expressing cells but not in control cells until lactate concentration reaches 100 mM. *C*, pertussis toxin (*PTX*; 100 ng/ml) inhibits GPR81 receptor activation in a GTPγS binding assay. Assay was performed in triplicate for each data point, and means ± S.E. are shown. *D*, L-lactate (10 mM) stimulates Erk phosphorylation in GPR81-expressing cells but not in control cells, which is inhibited by PTX (100 ng/ml).

TABLE 1

GPR81 from different mammalian species and their EC_{50} values for L-lactate

GPR81 coding regions from different species were PCR-amplified using the genomic DNAs from respective species and cloned into a mammalian expression vector pCIneo (Promega). The PCR primers used are listed in supplemental Table 1. The insert regions were sequenced to confirm the identities. The cloned genes were sequenced to confirm the sequence identities. A GTPγS binding assay using cell membranes expressing recombinant GPR81 was used to characterize L-lactate as the ligand for GPR81 from different species. The assays were performed in triplicates for each data point. EC_{50} values represent means ± S.E.

GPR81 from different species	GenBank™ accession number	Percentage of homology/identity to human GPR81	EC_{50} value for L-lactate
		%	mM
Human	EU809458	100/100	4.87 ± 0.64
Mouse long form	EU809459	87/81	6.73 ± 0.73
Mouse short form	EU809460	87/81	6.94 ± 0.84
Rat long form	EU809461	88/81	6.26 ± 0.92
Rat short form	EU809462	88/81	6.58 ± 0.81
Dog	EU809463	90/84	3.71 ± 0.47
Pig	EU809464	90/83	5.62 ± 0.68
Cow	EU809465	88/80	4.95 ± 0.42
Monkey	EU809466	97/95	4.05 ± 0.58

brain extract was lyophilized, redissolved in 1 mM HCl, and fractionated with a Restek AllureOA column. Iterative chromatography cycles resulted in a final separation and a single peak containing the active component (Fig. 1*B*). NMR structural analysis of the fraction with active component showed that both its ^{1}H and ^{13}C APT spectra are identical to that of L-lactate (Fig. 1*C*). The peak assignment is further supported by COSY and HSQC experiments (data not shown). The purity of the fraction was over 98% by both ^{1}H and ^{13}C NMR. Direct measurement of L-lactate contents in the tissue extracts shows that lactate content in the tissue extracts (supplemental Fig. 1) is consistent with the GPR81 ligand activities.

Pharmacological Characterization of L-Lactate as a Ligand for GPR81—To confirm that L-lactate is indeed an agonist ligand for GPR81, commercial L-lactate at various concentrations was tested for activation of GPR81 using both GTPγS binding and direct inhibition of cAMP accumulation. The results show that L-lactate stimulates GTPγS binding with an EC_{50} value of about 5 mM (Fig. 2*A* and Table 1) in cells transfected with human GPR81 (but not in control cells or cells expressing the niacin receptors (GPR109a or GPR109b)) (Fig. 2*A*). In SK-N-MC/CRE-β-gal cells (a cell line harboring a β-galactosidase gene under the control of the cAMP-responsive element) stably expressing GPR81, L-lactate inhibits forskolin-induced cAMP accumulation (EC_{50} = 4.16 ± 0.53 mM), whereas it has no effect in control SK-N-MC/CRE-β-gal cells (Fig. 2*B*). Both the GTPγS binding and inhibition of cAMP accumulation suggest that GPR81 is coupled to $G_{i/o}$ proteins. This hypothesis is supported by the observation that pertussis toxin inhibits L-lactate stimulated GTPγS binding (Fig. 2*C*) and Erk phosphorylation (Fig. 2*D*). Cells expressing GPR81 also showed higher basal Erk phosphorylation compared with control cells, and pertussis toxin treatment reduced the basal Erk phosphorylation (Fig. 2*D*). This may reflect either a constitutive level of activation of the receptor or an endogenous lactate tone within the cell/tissue, since essentially all cells produce L-lactate dependent upon metabolic conditions.

To investigate whether L-lactate stimulates the internalization of GPR81, we engineered a GPR81-expressing construct with a V5 tag fused to the N terminus of GPR81. V5-tagged-GPR81 responded appropriately to L-lactate stimulation as measured by GTPγS stimulation, and the dose-response curve was indistinguishable from the wild type receptor (supplemental Fig. 2). To detect the cell surface expression of V5-tagged GPR81, live cells were stained with anti-V5 antibody by diluting the antibodies into the cell culture medium. The results show that although no signal was detected in the control cells (Fig. 3*A*), clear cell surface staining of V5-GPR81 expression was detected from V5-GPR81-transfected cells (Fig. 3*B*). To measure receptor internalization, cells expressing V5-tagged GPR81 were first incubated with anti-V5 antibody, followed by L-lactate stimulation. The cells were then fixed and permeabilized,

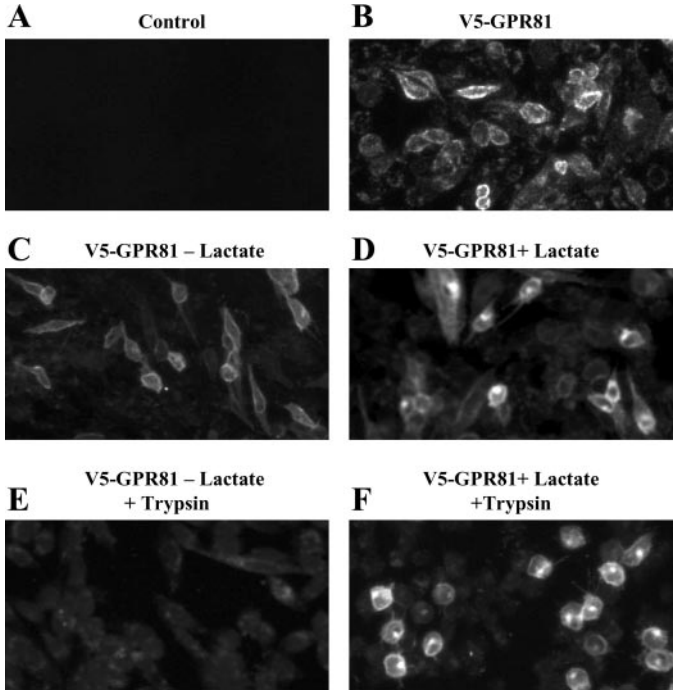

FIGURE 3. **L-Latate stimulates the internalization of GPR81.** V5-tagged human GPR81 was transiently expressed in CHO cells. Mock-transfected cells served as the control. Live cell staining with anti-V5 antibody diluted in cell culture medium followed by incubation with Cy3-labeled goat anti-mouse secondary antibody shows that no signal was detected in control (*A*), and V5-tagged GPR81 was expressed on the cell surface (*B*). V5-tagged GPR81 expressing cells were incubated with anti-V5 antibody diluted in cell culture medium first then treated either without (*C*) or with 25 mM L-lactate (*D*) for 30 min. The cells were then fixed, permeabilized, and stained with a Cy3-labeled goat anti-mouse secondary antibody, which will stain the bound anti-V5 antibodies both on the cell surface and internalized together with V5-tagged GPR81. In a parallel experiment, V5-GPR81-expressing cells were treated the same way as described in *C* and *D* but trypsinized before fixation and staining with the secondary antibody. The anti-V5 staining in cells without L-lactate treatment (*E*) was dramatically reduced, but the staining in L-lactate-treated cells (*F*) was not affected.

and the localizations of anti-V5 antibody-labeled receptors were detected with Cy3-labeled secondary antibody. The results show that L-lactate stimulates GPR81 redistribution in the cells. Compared with untreated cells (Fig. 3*C*), in L-lactate-treated cells, V5-GPR81 staining appeared at high intensity in intracellular organelles (Fig. 3*D*), indicating that L-lactate stimulates GPR81 internalization. Confirming this conclusion, in a parallel experiment, we treated the cells with trypsin following anti-V5 antibody incubation to remove the uninternalized antibody. The internalized anti-V5 antibody was then stained using a Cy3-labeled goat anti-mouse IgG antibody in the presence of permeabilization reagent. Our results show that although trypsin digestion reduces V5-GPR81 staining in cells without L-lactate treatment (Fig. 3*E*), the high intensity staining in L-lactate-treated cells appears in intracellular organelles and is resistant to trypsin digestion (Fig. 3*F*), indicating that they are internalized V5-GPR81.

To investigate whether L-lactate can also activate GPR81 from different mammalian species, the mouse and rat *GPR81* were cloned. Compared with the human *GPR81*, both mouse and rat *GPR81* coding regions are longer at the 5′-end, encoding an additional 8 amino acids at the N terminus. However, the ATG site corresponding to the human GPR81 translation initiation is still conserved in the mouse and rat *GPR81* cDNAs. Two clones for both mouse and rat *GPR81* were cloned, respectively, with one clone starting with the first ATG site (designated *GPR81L*) and another clone using the second ATG site (corresponding to the ATG site in the human *GPR81* translation initiation site, designated *GPR81s*). There was no observed difference between the pharmacology for the GPR81L and GPR81s clones (data not shown). Therefore, the GPR81L clones were used for all subsequent studies. We further cloned *GPR81* from monkeys, dogs, pigs, and cows. Overall, *GPR81* genes from different mammalian species are highly conserved (>80% sequence identity). The GenBank™ accession numbers for *GPR81* genes from different species are listed in Table 1. Pharmacological characterization of recombinant GPR81 from different species demonstrated that they all respond to L-lactate through stimulation of GTPγS incorporation in membranes (Table 1), indicating conservation of the phenomenon in different species.

TABLE 2

EC_{50} values and E_{max} of compounds tested as ligands for human GPR81

All compounds in solution were titrated to pH 7.4 using NaOH or HCl before testing as ligands for GPR81. GTPγS binding assay was used to characterize the compounds. EC_{50} values shown are means ± S.E. of triplicates.

Compounds	EC_{50} for GPR81	E_{max}
	mM	% of L-lactate
L-Lactate	4.87 ± 0.64	100
GHB	15.3 ± 2.14	110
DL-α-Hydroxybutyrate	8.51 ± 1.51	92
Glycolate	9.64 ± 1.35	85
Trifluoroacetate	5.41 ± 0.68	62
α-Hydroxyisobutyrate	7.83 ± 1.43	57
DCA	3.54 ± 0.57	35
DL-α-Hydroxycaproic acid	> 20	45[a]
Malate	> 20	35[a]
Tartrate	> 20	27[a]
D-Lactate	> 20	20[a]
Propionate	> 20	15[a]
Formiate	NA[b]	ND[c]
Acetate	NA[b]	ND
Pyruvate	NA[b]	ND
Citrate	NA[b]	ND
Butyrate	NA[b]	ND
DL-β-Hydroxybutyrate	NA[b]	ND
Succinate	NA[b]	ND
Niacin	NA[b]	ND
γ-Aminobutyrate	NA[b]	ND

[a] Agonistic activities have been observed for those compounds, but the EC_{50} values for those compounds are not calculated, because the dose-response curves for those compounds did not reach plateaus. The highest responses stimulated by those ligands (up to 50 mM) are shown as the percentage of the maximum response stimulated by L-lactate.

[b] NA, no activity. No agonistic activity was observed for these compounds (except niacin) when tested at concentrations up to 50 mM. Niacin was tested with the highest concentration of 10 mM.

[c] ND, not determined.

We next tested a series of related acids as ligands for GPR81 (Table 2). Our results show that α-hydroxybutyrate, glycolate, α-hydroxyisobutyrate, and γ-hydroxybutyrate are also low affinity agonists for GPR81. In contrast, D-lactate, α-hydroxycaproic acid, malate, tartrate, and propionate are weak partial agonists for GPR81, whereas niacin, pyruvate, β-hydroxybutyrate, acetate, γ-aminobutyric acid, and butyrate are not active (Table 2). L-Lactate was unable to activate either GPR109a or GPR109b (Fig. 2*A*). In addition, we found that dichloroacetate (DCA) and trifluoroacetate were capable of activating GPR81 (Table 2), albeit as partial agonists.

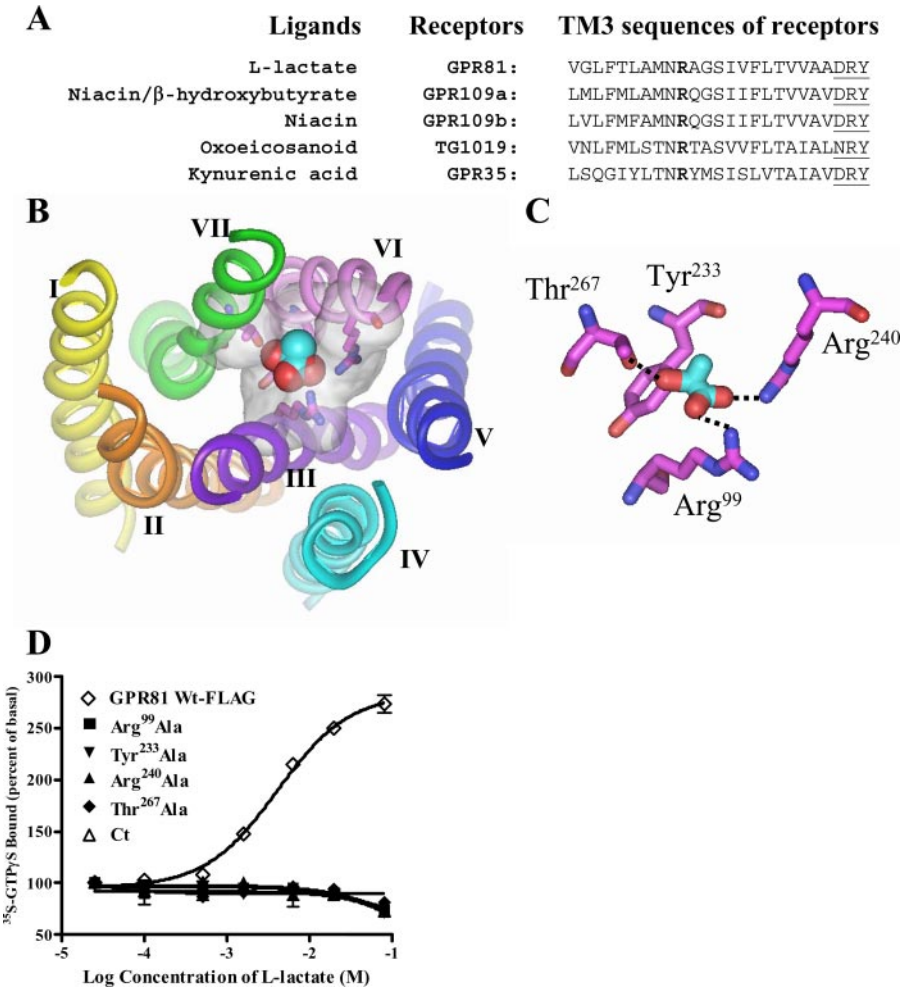

FIGURE 4. **The ligand-binding site of GPR81 for L-lactate.** *A*, TM3 amino acid sequence comparison among GPR81-related GPCRs. All sequences are from human receptors. The conserved Arg residues in TM3 are shown in *boldface type*. The DRY motifs in TM3 of the receptors are *underlined*. *B*, computer modeling of GPR81 ligand-binding site and likely interactions between GPR81 and L-lactate. The seven transmembrane helical domains of the GPR81 receptor are shown, with L-lactate (*space-filling representation*) docked in the putative binding site located between TM3, -6, and -7 (*purple*, *pink*, and *green*, respectively). The residues important for ligand binding are shown with a surface representation (Arg^{99}, Arg^{240}, Tyr^{233}, and Thr^{267}), depicting the overall shape of the binding site. *C*, close up view of the binding site. The carboxylate group of L-lactate (*cyan*) interacts with the two basic residues (Arg^{99} and Arg^{240}). The hydroxyl group of lactate interacts with the hydroxyl group of Thr^{267} and the Tyr^{233} lines the hydrophobic bottom of the binding pocket. *D*, site-directed mutagenesis studies of the human GPR81. Residues Arg^{99} in TM3, Tyr^{233} and Arg^{240} in TM6, and Thr^{267} in TM7 of the human GPR81 were mutated into Ala, respectively. The resulting receptors were expressed in CHO cells and characterized using L-lactate as the ligand in a GTPγS binding assay. The wild type receptor-expressing cells and the mock-transfected cells (*Ct*) were used as the controls. Each data point was analyzed in triplicate, and means ± S.E. are shown.

carboxyl group of these acid ligands. Supporting this hypothesis, mutation of this residue in GPR109a ($Arg^{111} \rightarrow$ Ala) abolished the receptor activity in response to niacin (21). Further studies (21) demonstrated that Arg^{251} at the TM6 of GPR109a is also important for its interaction with nicotinic acid. TM3, TM5, TM6, and TM7 are involved in the classic binding mode of GPCRs and their small molecule ligands (21–23). GPR81 shares high sequence similarity to niacin receptors GPR109a and GPR109b (66% homology and 52% identity at the amino acid sequence level), suggesting that GPR81 and GPR109a might have similar ligand-binding pockets. The fact that β-hydroxybutyrate is an agonist for GPR109a and binds to the same site as niacin (4), whereas the GPR81 ligands, such as L-lactate and α-hydroxybutyrate, are structurally very similar to β-hydroxybutyrate further suggests that L-lactate binds to GPR81 in a similar mode as β-hydroxybutyrate does to GPR109a. Receptor homology modeling of human GPR81 shows a likely L-lactate interaction with GPR81 in the classical GPCR small molecule putative binding site, similar to what has been proposed for niacin/β-hydroxybutyrate-GPR109a binding. The carboxyl group of L-lactate probably interacts with Arg^{99} of in the TM3 (corresponding to the Arg^{111} of GPR109a), whereas Tyr^{233} (corresponding to the highly conserved Trp in all family A GPCRs), Arg^{240} (corresponding to Arg^{251} of GPR109a) in TM6, and Thr^{267} in TM7 also participate in the ligand interactions (Fig. 4, *B* and *C*). To confirm this hypothesis, we made mutations at these positions in the human GPR81, and our results showed that each of R99A, Y233A, R240A, and T267A mutations diminished the response of GPR81 to L-lactate (Fig. 4*D*). The expression levels of the all GPR81 mutants are comparable with that of the wild type receptor (supplemental Fig. 3).

The Ligand-binding Site of GPR81—Previous studies have demonstrated that an Arg residue in the third transmembrane domain (TM3) of the succinate receptor (GPR91) and the α-ketoglutarate receptor (GPR99) is involved in the ligand/receptor interaction of the carboxyl group of their acid ligands succinate and α-ketoglutarate. Mutation of this Arg residue abolishes the receptor activity in response to these acid ligands (18). Comparison of a few closely related small organic acid receptors, including kynurenic acid receptor (GPR35) (19), oxoeicosanoid receptor TG1019 (20), and niacin receptors GPR109a and GPR109b, shows that they all share a highly conserved Arg residue in their TM3 domain (Fig. 4*A*), suggesting that this Arg residue in these receptors might be involved in their receptor ligand interactions, possibly through the interaction with the

Tissue Expression of GPR81—To gain a clearer perspective of the potential of L-lactate as an endogenous ligand for GPR81, we examined the tissue expression profile of GPR81. cDNAs from different human, rat, or mouse tissues as well as 3T3-L1 cells (a mouse cell line) were amplified for *GPR81* mRNA by quantitative PCR using specific primers for human, rat, or mouse *GPR81*, respectively. As internal controls, specific prim-

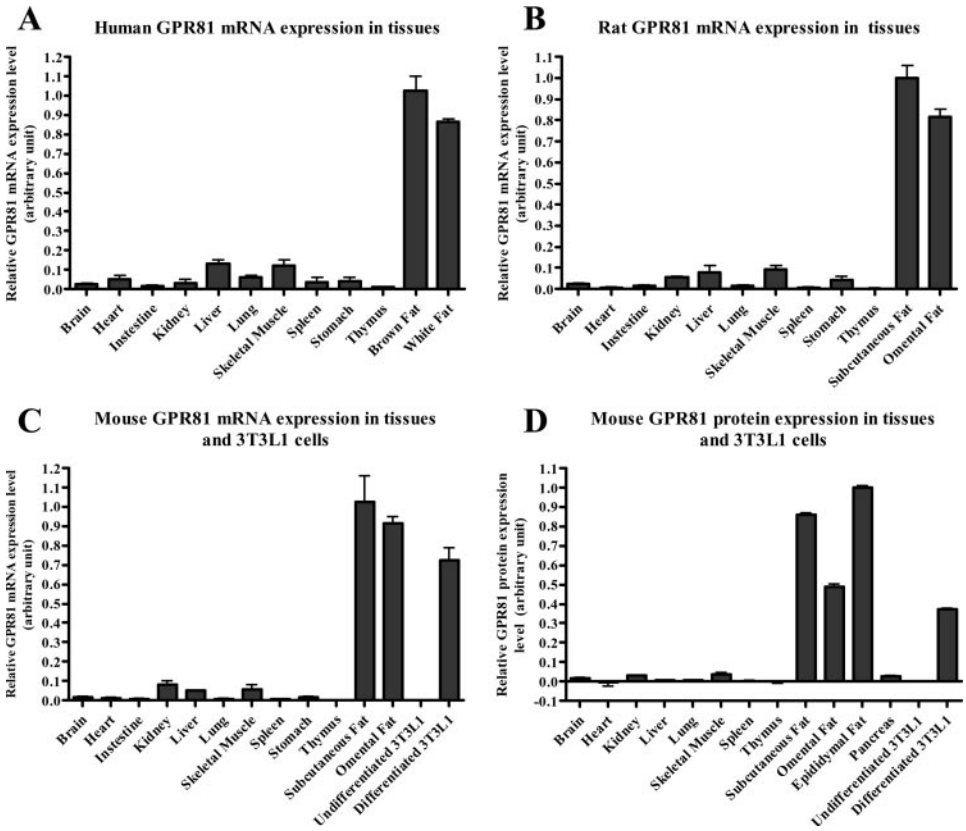

FIGURE 5. **GPR81 mRNA (*A–C*) and protein (*D*) expression in human (*A*), rat (*B*), and mouse (*C* and *D*) tissues or cells.** The mRNA expression was analyzed by quantitative RT-PCR. *GPR81* mRNA expression was normalized to the mRNA expression of β-actin. The mRNA expression level for the highest in each species was arbitrarily set at 1. The PCR primers used to amplify *GPR81* or β-actin mRNAs are listed in supplemental Table 2. Each sample was analyzed in triplicate, and means ± S.E. are shown. To detect the mouse GPR81 protein expression, crude plasma membranes were prepared from different mouse tissues (dissected from wild type or *GPR81*-deficient mice) or cultured cells and solubilized by Triton X-100. A sandwich ELISA, using a chicken anti-mouse GPR81 as the capture antibody and a rabbit anti-mouse GPR81 antibody as the detection antibody, was employed to measure GPR81 protein expression in different tissues or cells. Recombinant mouse GPR81 protein expressed in COS-7 cells was diluted in different concentrations to serve as the standard to quantify the relative GPR81 protein expression levels. The GPR81 protein expression levels detected from different mouse tissues and cells were normalized by the tissue weights or cell pellet weights. The highest GPR81 protein expression level for all samples was arbitrarily set at 1. Each sample was analyzed in triplicate, and means ± S.E. are shown. No positive signals were detected from all tissues from *GPR81*-deficient mice.

ers for human, rat, or mouse β-actin were used to amplify the β-actin mRNA from the same samples. Pure PCR products for *GPR81* (human, rat, or mouse) and actin (human, rat, or mouse) were diluted at different concentrations and used as standards for quantification. The relative abundance of *GPR81* mRNA was normalized to the expression level of β-actin. The results show that in humans, *GPR81* is most abundantly expressed in brown fat, with high expression in white fat. It is also detectable at lower levels in liver, kidney, skeletal muscle, brain, and many other tissues (Fig. 5*A*). Similarly, in rats, *GPR81* is found highly expressed in subcutaneous fat and omental fat and detectable in lower levels in brain and many other tissues (Fig. 5*B*). A similar expression pattern of *GPR81* was observed in mouse tissues (Fig. 5*C*). In addition, *GPR81* mRNA is highly expressed in differentiated 3T3-L1 adipocytes (Fig. 5*C*). This is consistent with previous findings from other investigators (3, 9). To study the GPR81 protein expression in tissues, we generated multiple antibodies against mouse GPR81 either from rabbit or chicken. Testing of these antibodies by Western blot using recombinant mouse GPR81 protein showed that by SDS-PAGE, the mouse GPR81 protein formed large aggregates and the Western blot signal spread from 50 to 250 kDa and higher (supplemental Fig. 4, *A–C*), which made it impractical to detect and quantify the endogenous GPR81 protein (normally expressed at much lower levels compared with the recombinant system). Immunostaining using those antibodies demonstrated specific staining in cells recombinantly expressing mouse GPR81 (supplemental Fig. 4, *D–G*) but showed a similar pattern of signal on tissue sections from both of the wild type and *GPR81*-deficient mice (data not shown) and offered no useful information regarding the endogenous GPR81 protein expression pattern. To improve the selectivity and sensitivity of the protein expression analysis, we established a sandwich ELISA method using a chicken anti-mouse GPR81 as the capture antibody and a rabbit anti-mouse GPR81 antibody as the detection antibody. The double selection of the two antibodies made the assay extremely specific for mouse GPR81. Our results showed that GPR81 protein was detected at the highest levels in mouse epididymal and subcutaneous fats, with slightly lower level in omental fat. Low levels of GPR81 protein expression were detected in the brain, skeletal muscle, kidney, liver, and the pancreas (Fig. 5*D*). In addition, we also demonstrated that differentiated 3T3-L1 cells also express GPR81 protein (Fig. 5*D*). No signal was detected from any tissues from the *GPR81*-deficient mice (data not shown).

Functional Study of GPR81—Given that the predominant expression of GPR81 is in fat tissues and adipocytes and that there is a strong structural and functional similarity to the niacin receptor GPR109a, we investigated whether L-lactate, acting through GPR81, could also modulate lipolysis. During lipolysis in adipocytes, fat (lipid) is hydrolyzed into fatty acids and glycerol. Lipolysis in adipocytes is regulated by cAMP levels. Ligands such as adrenaline and β-adrenergic agonists (*e.g.* isoproterenol) that stimulate cAMP in adipocytes facilitate lipolysis. In contrast, activation of the insulin receptor by insulin (which induces phosphodiesterase and the subsequent hydrolyzation cAMP) or activation of G_i-coupled G-protein coupled receptors (which inhibits cAMP synthesis) inhibit lipolysis. Our results show that similar to niacin and insulin (24), L-lactate also inhibits glycerol and fatty acid release in differentiated 3T3-L1 cells (Fig. 6, *A* and *B*). Similarly, L-lactate was also capable of

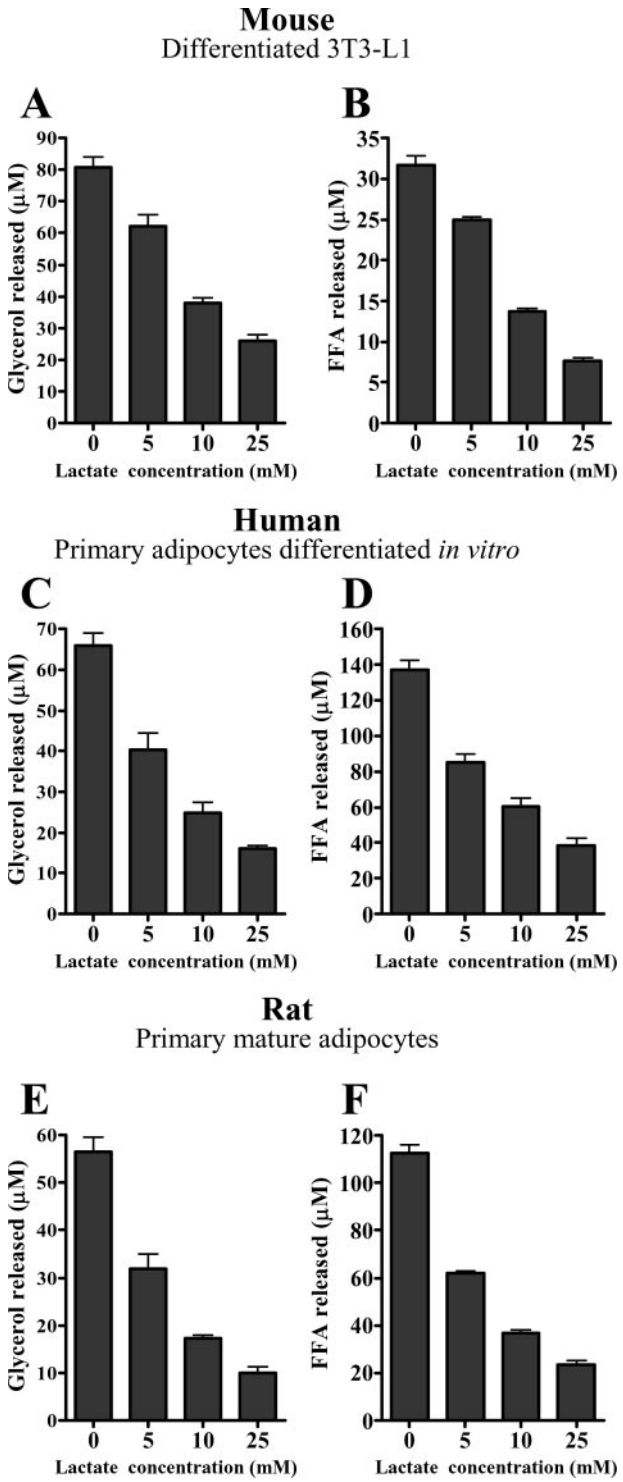

FIGURE 6. **Antilipolytic effect of L-lactate on adipocytes.** L-Lactate inhibits glycerol (*A*, *C*, and *E*) and free fatty acid (*B*, *D*, and *F*) release in differentiated 3T3-L1 cells (*A* and *B*), human subcutaneous adipocytes differentiated *in vitro* (*C* and *D*), and primary mature adipocytes from rat epididymal fat (*E* and *F*). Each data point was assayed in triplicate, and means ± S.E. are shown.

inhibiting lipolysis in primary human subcutaneous adipocytes differentiated *in vitro* (Fig. 6, *C* and *D*). Very similar results have been observed in primary mature adipocytes isolated from rat epididymus (Fig. 6, *E* and *F*). These data indicate that L-lactate is a mediator of lipolysis in adipocytes and strongly suggest that L-lactate exerts its antilipolytic function through GPR81. To confirm that GPR81 is involved in lipolysis and determine if the relationship held true between L-lactate and GPR81 *in vivo*, we obtained a *GPR81* knock-out mouse line from Deltagen (*GPR81*$^{-/-}$). *GPR81*$^{-/-}$ mice were viable and fertile and displayed no overt differences from wild type. Mature adipocytes from the subcutaneous fat of female mice were released from the fat tissues by digestion with collagenase and used for the study of glycerol and FFA release as a reflection of basal lipolysis. The results showed that L-lactate dose-dependently inhibited glycerol and FFA release in adipocytes from the wild type mice (Fig. 7, *A* and *B*). In the same experiment, niacin, insulin, and propionate also inhibited glycerol release. In contrast, adipocytes from *GPR81*$^{-/-}$ mice were unresponsive to all concentrations of L-lactate but responsive to insulin, propionate, and niacin (Fig. 7, *A* and *B*). Very similar results were observed in subcutaneous and epididymal adipocytes from the male mice (data not shown). Surprisingly, we observed that the efficacy of niacin in the *GPR81*$^{-/-}$ mouse adipocytes is partially reduced. We have already shown that niacin is not capable of activating recombinant GPR81. To determine whether *GPR109a* (the *Puma-G* gene is the mouse counterpart of the human gene *GPR109a*) is intact in *GPR81*$^{-/-}$ mice or its expression was affected during the creation of the *GPR81* knock-out, we conducted some PCR experiments. Examination of the *Puma-G* gene from *GPR81*$^{-/-}$ mouse genomic DNA showed that the complete coding region (1 kb) of *Puma-G* gene is intact (Fig. 8*A*), which is further confirmed by direct sequencing of the PCR product. In parallel, we also analyzed the *GPR81* gene in both wild type and *GPR81*$^{-/-}$ mice and found that the wild type *GPR81* gene (1 kb) is intact. For *GPR81*$^{-/-}$ mice, no intact *GPR81* gene is detectable, and only the disrupted *GPR81* gene (8 kb) is observed. DNA sequencing of the *GPR81* PCR product confirms disruption of the *GPR81* gene in the *GPR81*$^{-/-}$ mice, including the deletion of the TM2 coding region of the *GPR81* gene and the replacement of that region with the *lacZ* gene and a *neo* gene (Fig. 8*A*). Quantitative RT-PCR analysis of the *Puma-G* mRNA expression in the fat tissues showed that it is expressed in subcutaneous fat (Fig. 8*B*) and epididymal fat (data not shown) of both the wild type and *GPR81*$^{-/-}$ mice, and we have not observed a significant difference between the wild type and *GPR81*$^{-/-}$ mice. We also demonstrated that DCA as well as trifluoroacetate, which are both agonists for GPR81, could suppress lipolysis in adipocytes from wild type mice, whereas this effect was partially reversed in the *GPR81*$^{-/-}$ mice. This suggests that DCA and trifluoroacetate may suppress lipolysis through GPR81; however, we have also observed that DCA is capable of activating GPR41, but not GPR43 or PUMA-G, whereas trifluoroacetate can activate both GPR41 and PUMA-G (but not GPR43) at high concentrations (supplemental Fig. 5). This finding in part helps to explain why the antilipolytic effects of DCA and trifluoroacetate are only partially reversed; however, we cannot rule out a more complex explanation similar to that for niacin.

DISCUSSION

We have shown that L-lactate is capable of activating a previously known orphan GPCR, GPR81. GPR81 is highly expressed in differentiated 3T3-L1 adipocytes and primarily

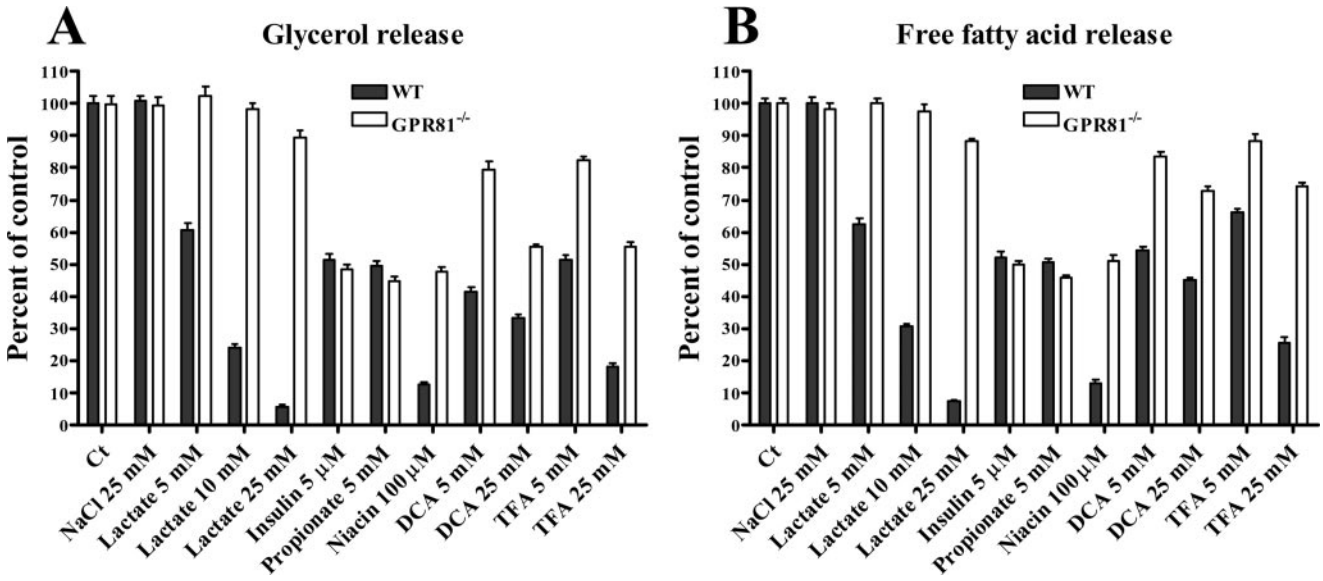

FIGURE 7. **Adipocytes from *GPR81*-deficient mice lack antilipolytic response to L-lactate.** L-Lactate inhibits glycerol (*A*) and free fatty acid (*B*) release in adipocytes from wild type (*WT*) mice but not in *GPR81*$^{-/-}$ mice. Different reagents were added to the isolated adipocytes to inhibit lipolysis. Glycerol (*A*) and FFA (*B*) release were measured from the adipocyte incubation buffer after 2 h of incubation. The glycerol (154 μM for WT and 125 μM for *GPR81*$^{-/-}$) and FFA (337 μM for the WT and 270 μM for *GPR81*$^{-/-}$) release from the control groups were set as 100%, and the results from other groups are presented as a percentage of the respective controls. Each data point was assayed in triplicate and means ± S.E. are shown.

expressed in adipose tissues. L-Lactate inhibits FFA and glycerol release in differentiated 3T3-L1 adipocytes and adipocytes from subcutaneous and epididymal fat *in vitro*. It is well established that L-lactate is produced during intense exercise, and it was demonstrated over 30 years ago that lactate suppresses lipolysis in exercising humans. Boyd *et al.* (25) hypothesized that plasma lactate produced during intense exercise is a direct signal to fat to decrease lipolysis during a time when other factors are trying to increase the further release of FFAs. A physiological rationale for this feedback is to limit the supply of FFAs (which have a greater oxygen requirement per mole of ATP produced than pyruvate) during times when glycolysis rates exceed mitochondrial respiration rates. In support of this hypothesis, Achten and Jenkendrup (26) demonstrated that a rise in plasma lactate during exercise is highly correlated with the reduction in FFA oxidation. GPR81 responds to lactate in the precise physiological range induced during intense exercise. In contrast, Fain and Shepherd (27) reported that 3 mM lactate was able to inhibit adenylate cyclase in rat white fat cells but had no effect on lipolysis. Ferrannini *et al.* (28) reported the infusion of lactate (up to 2.4 mM) in human volunteers but found no significant effect on lipolysis. Both of these studies used concentrations of lactate at the lower end of the dose-response curve that we have observed for recombinant GPR81. De Pergola *et al.* (29) reported in 1989 that lactate could suppress stimulated but not basal lipolysis and that this effect was only significant when lactate reached 16 mM. Lower exposure was not described in the paper. In addition to exercise, L-lactate can be produced by various tissues, particularly fat, after a metabolic load (30, 31). In the presence of insulin and glucose, when fat cells take up glucose, they also release L-lactate (31). We demonstrated that *in vitro*, 3T3-L1 adipocytes, when glucose and insulin are present, release lactate (supplemental Fig. 6). Thus, L-lactate may be serving as an autocrine ligand for adipocytes, signaling the need to suppress lipolysis, when glucose is abundant. We propose GPR81 to be the receptor responsible for responding to endogenous increases in plasma lactate levels, whether they come from intense exercise or exposure to a metabolic load. Thus, GPR81 may represent another target in the growing array of metabolic pathways for the treatment of dyslipidemia and other metabolic disorders.

Is L-Lactate an Endogenous Physiological Ligand for GPR81?—It is of significance to note that the EC_{50} value for L-lactate to activate GPR81 is extremely high (~5 mM) compared with values typically associated with G-protein-coupled receptors, which are often found in the nanomolar range. One obvious question is whether L-lactate can be a physiological ligand for GPR81. To be an endogenous physiological ligand for a receptor, the ligand needs to be present at the site of action (where its receptor is located) and reach effective concentration ranges. In addition, to be a regulator of receptor activation, the EC_{50} value of an endogenous physiological ligand for its receptor should be in the dynamic range of the ligand concentration under physiological conditions. Under these conditions, the concentration change of the ligand should be readily sensed by its receptor and reflected by receptor activation. In humans, resting L-lactate concentrations in the plasma are in the 0.5–2 mM range, whereas L-lactate levels after intense anaerobic exercise can reach upwards of 10–20 mM (32–35). Additionally, after a metabolic load, such as a meal, adipocytes produce L-lactate as well, which will increase the local lactate concentration in fat tissues (31). This places the observed GPR81 EC_{50} of 3–5 mM in the middle of the dynamic physiological range of the putative endogenous ligand, L-lactate. Of the other acids that showed agonism for GPR81, all were lower affinity than L-lactate, and none of them are known to exist *in vivo* at concentrations that would be relevant to their EC_{50} values for GPR81. Therefore, L-lactate remains the most attractive candidate as the endoge-

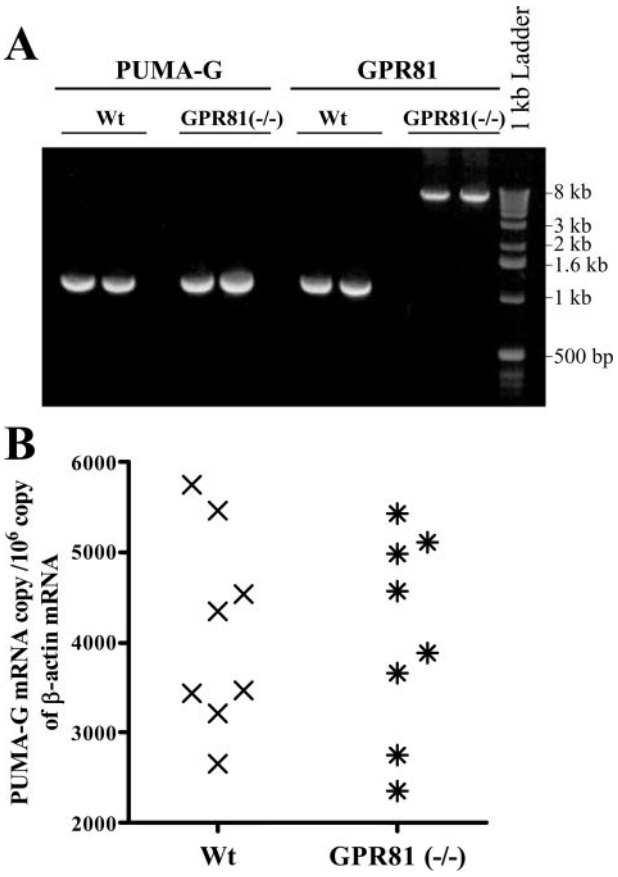

FIGURE 8. **PCR analysis of mouse niacin receptor *Puma-G* gene and mRNA expression in *GPR81*-deficient mice.** *A*, PCR analysis of *Puma-G* and L-lactate receptor (GPR81) genes using the genomic DNAs from the wild type (*Wt*) mice and *GPR81* knock-out mice. *Puma-G* primers and *GPR81* primers (supplemental Table 3) that amplify the entire coding regions of *Puma-G* and *GPR81* genes, respectively, were used. PCR results from two wild type mice and two *GPR81*$^{-/-}$ mice are shown. The PCR products were sequenced to verify the identities of the *Puma-G* gene and the wild type *GPR81* gene and the knock-out of *GPR81* in *GPR81*$^{-/-}$ mice. *B*, quantitative RT-PCR analysis of *Puma-G* mRNA expression in the subcutaneous fat from the wild type and *GPR81*$^{-/-}$ knock-out mice. Subcutaneous fat samples from eight male wild type mice and eight male *GPR81*$^{-/-}$ mice were analyzed for *Puma-G* mRNA expression by quantitative RT-PCR using *Puma-G*-specific primers (supplemental Table 2). The β-actin mRNA expression was used as the internal standard. The *Puma-G* mRNA expression is normalized to the level of β-actin mRNA expression.

nous ligand for GPR81. One means by which to appreciate the significance of a finding is to determine if it is evolutionarily conserved. Since L-lactate is such a fundamental and common metabolite in many diverse animal species, we hypothesized that if lactate is serving as a physiological ligand for GPR81 as a signaling molecule, the L-lactate/GPR81 ligand/receptor pair relationship should also be conserved. The fact that GPR81 from mice, rats, pigs, cows, dogs, and monkeys all respond to L-lactate at the physiological L-lactate concentration indicates that the function of the GPR81 is conserved among those species, and these results offered strong support that L-lactate is indeed the endogenous physiological ligand for GPR81. As we expected, the antilipolytic response to lactate was absent in adipocytes from *GPR81*-deficient mice. However, it was unexpected that adipocytes from *GPR81*-deficient mice would demonstrate a reduced response to niacin. GPR81 and PUMA-G are both expressed on mouse adipocytes and are highly related to one another structurally. It is possible that the two receptors may have direct interactions, and the reduced niacin effect in the *GPR81*$^{-/-}$ mice might hint that the full niacin receptor function is dependent on GPR81 expression. In our recombinant systems, lactate does not activate GPR109a and niacin does not activate GPR81, and co-expression of the two receptors does not change their respective pharmacology (data not shown). Therefore, direct evidence for a receptor-receptor interaction is lacking and thus will require additional experimentation to understand why niacin is slightly less effective in the *GPR81*$^{-/-}$ mice, despite the presence of the *Puma-G* mRNA.

Is L-Lactate an Allesteric Modulator of the Constitutive Activity of GPR81?—The high EC_{50} value (~5 mM) of L-lactate to stimulate GPR81, which is unusual for most ligand-GPCR pairs, combined with the high basal activity of Erk phosphorylation in cells recombinantly expressing GPR81, leads to speculation as to whether L-lactate is an allosteric modulator to modulate the basal activity of GPR81. To specifically address this question, we took two approaches. The first was to determine whether we could use site-directed mutagenesis and computer modeling to identify a *bona fide* ligand binding domain for lactate in the receptor. The second was to examine whether lactate was capable of inducing receptor internalization, which is a phenomenon strongly associated with direct receptor activation and not allosteric modulation. Regarding the binding site of L-lactate to GPR81, the similarities between GPR81 and GPR109a as well as that between L-lactate/α-hydroxybutyrate and β-hydroxybutyrate strongly suggest that L-lactate/α-hydroxybutyrate could bind and activate GPR81 in a fashion similar to how β-hydroxybutyrate/niacin bind/activate GPR109a. Computer modeling suggests that L-lactate could interact with the Arg^{99} in TM3, Tyr^{233}, and Arg^{240} in TM6 and Thr^{267} in TM7, which would be analogous to how niacin/β-hydroxybutyrate binds to GPR109a. This hypothesis is strongly supported by our site-directed mutagenesis studies. Specific mutation of any of these key residues in the transmembrane domain regions significantly reduced the ability of lactate to activate GPR81. These results strongly suggest that L-lactate binds to GPR81 in a classical small molecule ligand/GPCR binding fashion instead of serving as a allosteric modulator. Additionally, when true ligands bind and activate GPCRs, they usually initiate a series of G-protein dependent events, one of which is the initiation of receptor internalization. Receptor internalization can be measured a number of ways. We chose to create a chimeric receptor with a fused N-terminal V5 epitope. Upon ligand-induced receptor activation, the subsequent internalization of the receptor can be monitored using an anti-V5 antibody that is preincubated with the cells. Using this methodology, we were able to demonstrate that L-lactate could induce the internalization of GPR81. Taken together with ligand binding domain modeling and site-directed mutagenesis, the lactate-induced internalization provides strong evidence that lactate is in fact acting as a direct agonist for GPR81 rather than acting as an allosteric modulator. This is also supported by the observation that in washed membrane homogenates, lactate is capable of directly stimulating GTPγS incorporation into G-proteins in the absence of other ligands.

Is GPR81 Responsible for Some of the in Vivo Function of DCA?—DCA is a known inhibitor of pyruvate dehydrogenase kinase and is commonly found in chlorinated drinking water. Because of its known mechanism of action, high doses of DCA have been used clinically to treat lactic acidosis (36–38) via lactate suppression, and it is also currently in clinical trials for the treatment of cancer (39–41). In addition, DCA has been shown to stimulate glycogen synthesis in isolated primary hepatocytes (42) in the liver (43) and muscle (44) after oral administration and reported to stimulate insulin secretion and lower glucose concentration in the periphery after intracerebra ventricular administration (45), which is very similar to that of L-lactate (45). It is important to note that the concentration for DCA is often very high when administered *in vivo* (100 mg/kg to 1 g/kg) or used in the cell culture (1–10 mM), which suggests that the DCA concentrations might be sufficient to activate GPR81. It is therefore conceivable that some of the clinical effects of DCA could be due to the activation of GPR81.

Is GPR81 the γ-Hydroxybutyric Acid (GHB) Receptor?—GHB is a putative neuromodulator that has profound physiological, pharmacological, and biochemical effects in the brain and the periphery (46). The functions of GHB in the brain include anxiolytic effect, sleep modulation, anesthesia, and absence seizures. In addition, GHB regulates the neurotransmitter release of dopamine, endogenous opioids, glutamate, and acetylcholine. GHB has been used as an anesthetic agent, for treating sleep disorders, and for alcohol dependence (for reviews, see Refs. 47 and 48) and is currently marketed as Xyrem® for the treatment of cataplexy associated with narcolepsy. In 1989, GHB was sold over the counter as a bodybuilding and fat-burning compound. Due to its euphoric effects, high abuse potential, and notoriety as the "date rape drug," GHB was banned in 1990 for nonprescription sale, and in 2000 GHB was classified as a schedule I drug by the Food and Drug Administration. The precise mechanism of action by GHB remains unknown. Although there is clear evidence for an interaction with the γ-aminobutyric acid B receptor, specific and distinct binding sites for GHB in the brain continue to suggest that GHB has additional pharmacological targets. However, no specific molecular entity accountable for GHB receptor activity has been so far identified. The finding that L-lactate and other α-hydroxy acids activate GPR81 led us to test whether GHB and the GPR109a agonist β-hydroxybutyrate are also capable of activating GPR81. Although we found that β-hydroxybutyrate is inactive at GPR81, GHB is actually a full agonist for human GPR81 although with very low potency (EC_{50} = 15 mM). Further evaluation shows that GHB also activates mouse and rat GPR81s with EC_{50} values at 3.5 and 5 mM, respectively. Endogenous concentrations of GHB in human urine have been reported in the 1–20 μM range (49). Acute systemic exposure after administration of 4.5 g of GHB in humans yields a plasma C_{max} of ~1 mM (50, 51). Elliott (52) examined several nonfatal cases of GHB overdose and observed plasma concentrations ranging from ~1 to 5 mM, so it is unlikely that endogenous GHB can ever approach the exposure needed to activate GPR81. However, it is conceivable to achieve exogenous concentrations that may be capable of GPR81 activation, although the concentration would presumably be extremely dangerous.

In summary, we identified L-lactate as an endogenous ligand for GPR81 and a mediator of lipolysis in adipocytes through GPR81. Interestingly, L-lactate/GPR81 together with recently identified ligand receptor pairs, such as β-hydroxybutyrate/GPR109a, propionate/GPR41 (53), and acetate/GPR43 (53), all appear to play a similar role on adipocytes through negative regulation of adenylate cyclase, resulting in suppression of lipolysis in response to small acidic metabolic substances. Additionally, other recently identified ligand/receptor pairs, such as succinate/GPR91, α-ketoglutarate/GPR99, bile acids/TGR5, and LCFA/GPR120, demonstrate that substances that have traditionally been thought of as fuel, waste, or metabolic intermediates actually are directing physiology through cellular signaling. Our identification of lactate as an endogenous ligand for GPR81 puts one more piece of the puzzle in place. It helps to answer one of critical questions about the role of lactate in signaling to fat metabolism that has been asked over the past 40 years. Additionally, it provides an attractive target for the treatment of dyslipidemia.

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