(D)-β-Hydroxybutyrate Inhibits Adipocyte Lipolysis via the Nicotinic Acid Receptor PUMA-G

Received for publication, May 23, 2005
Published, JBC Papers in Press, June 1, 2005, DOI 10.1074/jbc.C500213200

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As a treatment for dyslipidemia, oral doses of 1–3 grams of nicotinic acid per day lower serum triglycerides, raise high density lipoprotein cholesterol, and reduce mortality from coronary heart disease (Tavintha-ran, S., and Kashyap, M. L. (2001)Curr. Atheroscler. Rep. 3, 74–82). These benefits likely result from the ability of nicotinic acid to inhibit lipolysis in adipocytes and thereby reduce serum non-esterified fatty acid levels (Carlson, L. A. (1963)Acta Med. Scand. 173, 719–722). In mice, nicotinic acid inhibits lipolysis via PUMA-G, a Gαi3-coupled seven-transmembrane receptor expressed in adipocytes and activated macrophages (Tunaru, S., and Kashyap, M. L. (2001)Curr. Atheroscler. Rep. 3, 74–82). These benefits likely result from the ability of nicotinic acid to inhibit lipolysis in adipocytes and thereby reduce serum non-esterified fatty acid levels (Carlson, L. A. (1963)Acta Med. Scand. 173, 719–722). In mice, nicotinic acid inhibits lipolysis via PUMA-G, a Gαi3-coupled seven-transmembrane receptor expressed in adipocytes and activated macrophages (Tunaru, S., and Kashyap, M. L. (2001)Curr. Atheroscler. Rep. 3, 74–82). These benefits likely result from the ability of nicotinic acid to inhibit lipolysis in adipocytes and thereby reduce serum non-esterified fatty acid levels (Carlson, L. A. (1963)Acta Med. Scand. 173, 719–722).

For the generation of stable cell lines, 5 × 10⁶ CHO-K1 cells were transfected with 12 μg of plasmid DNA (pCDNA3.1, Invitrogen) containing either HM74a, HM74, or PUMA-G expressed from the cytomeg-alovirus promoter. Two days after transfection, the growth medium was supplemented with 400 μg/ml G418 to select for antibiotic-resistant cells. Clonal CHO-K1 cell lines that stably express HM74a, HM74, or PUMA-G were selected based on the ability of nicotinic acid (HM74a and PUMA-G) or ST11589 (an Arena HM74-specific agonist; data not shown) to inhibit forskolin-induced cAMP production.

Calcium Mobilization—CHO-K1 cells expressing an NFAT-β-lactamase reporter and the promiscuous Ga subunit Gαi3 (kind gift of K. Sullivan, Merck Research Laboratories) were stably transfected with either empty vector (pCDNA3.1, Invitrogen) or vector expressing PMAG-HM74a, HM74, or HM74. Cells were seeded at 10,000 cells/well in 384-well culture plates and grown overnight at 37 °C. 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM t-glutamine, 10 mM HEPES, pH 7.4, 0.1 mM MEM non-essential amino acids solution, 1 mM sodium pyruvate, 0.6 μg/ml hygromycin B, 0.5 μg/ml zeocin, and 1 mg/ml genetin (BD Biosciences). Cells were washed four times with Hanks’ balanced salt solution containing 10 mM HEPES, pH 7.4, and loaded with calcium-sensitive dye by incubating with an equal volume of Molecular Devices calcium assay kit loading buffer at 37 °C for 1 h. Calcium response in the fluorometric imaging plate reader assay was measured according to the directions from Molecular Devices.

The abbreviations used are: AcAc, acetoacetate; (D)-β-OHB, (D)-β-hydroxybutyrate; NEFA, non-esterified fatty acid; GTPγS, guanosine 5’-O-(3-thiotriphosphate).

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(b)-β-Hydroxybutyrate Inhibits Lipolysis via PUMA-G

RESULTS

To ask whether β-OHB is a ligand for HM74a, we used Chinese hamster ovary (CHO) cells that stably express the chimeric G-protein α subunit G_q5 (18) and harbor either a control vector or vectors that expresses either HM74a or its paralog HM74, which is 95% identical at the amino acid level to HM74a but has ~1000-fold less affinity for nicotinic acid (4, 5).

Use of G_q5 allows the normally G_o-coupled HM74 and HM74a to signal via the G_q pathway leading to Ca^{2+} mobilization.

Nicotinic acid elicited Ca^{2+} mobilization only in cells expressing HM74a (Fig. 1A). In contrast, Acifran, an agonist on both HM74 and HM74a (4), elicited a response from both receptors demonstrating that HM74 was functional and could be used as a specificity control in this assay. Given that ketone bodies can reach millimolar concentrations in serum, we tested these compounds at 15 mM. Both (D)- and (L)-β-OHB, but not acetocetate or free acetone, elicited a Ca^{2+} response in cells expressing HM74a but not HM74. Whereas the D-isomer of β-OHB is the sole form encountered in high concentrations physiologically (7), both D- and L-isomers have been shown to inhibit lipolysis in vitro (17), consistent with the results shown here. Similar results were observed for cells expressing murine PUMA-G (data not shown).

Next, we determined the half-maximal concentration (EC_{50}) of β-OHB required to stimulate receptor-mediated guanine nucleotide exchange on Go using a [35S]GTPyS binding assay with membranes prepared from untransfected CHO cells or CHO cells stably expressing either mouse PUMA-G, human HM74a, or HM74 (Fig. 1B and Table I). Nicotinic acid stimulated [35S]GTPyS binding only in membranes from cells expressing HM74a (EC_{50} = 104 ± 5 nM) or the mouse ortholog PUMA-G (EC_{50} = 43 ± 3 nM), whereas Acifran was active on all three receptors (EC_{50} = 1127 ± 59 nM for HM74a, 358 ± 30 nM for PUMA-G, and 7039 ± 586 nM for HM74). Racemic (±)-β-OHB also showed some degree of activity on all receptors; however, it was more potent on HM74a (EC_{50} = 0.8 ± 0.06 mM) and its ortholog PUMA-G (EC_{50} = 0.7 ± 0.04 mM) than on HM74. The EC_{50} for the l-enantiomer was ~2-fold higher than that of the physiologically relevant d-enantiomer (Table I). Both the sodium and lithium salts of β-OHB were active, indicating that the anion is the active component. Moreover, sodium salts of other small monocarboxylic acids with similar pK_a values to β-OHB (α-hydroxybutyrate and lactate) were not significantly active in this assay (Table I). At high concentrations, lithium acetocetate, but not acetone or lithium chloride, elicited [35S]GTPyS binding to all three receptors but not to membranes from untransfected cells, suggesting that acetocetate is a weak agonist of these receptors.
Ligand-induced \(^{(35)S}\)GTP-\(\gamma\)S binding to membranes from CHO cells expressing PUMA-G, HM74a, or HM74

All compounds listed were inactive on membrane from untransfected CHO cells (data not shown). Data represent the mean values \(\pm\) S.E. for \(\geq 3\) assays. Inactive, no response at highest concentration tested.

| Compound                        | PUMA-G       | HM74a         | HM74        |
|---------------------------------|--------------|---------------|-------------|
| Nicotinic acid                  | 0.04 \(\pm\) 0.003 | 0.10 \(\pm\) 0.005 | \(>100^a\) |
| Acifran                         | 0.36 \(\pm\) 0.03  | 1.13 \(\pm\) 0.06 | 7.04 \(\pm\) 0.6 |
| Lithium (\(\text{L},\text{L}\))-\(\beta\)-hydroxybutyrate | 727 \(\pm\) 40  | 793 \(\pm\) 60 | \(>25,000\) |
| Sodium (\(\text{D},\text{D}\))-\(\beta\)-hydroxybutyrate | 518 \(\pm\) 37  | 767 \(\pm\) 57 | \(>25,000\) |
| Sodium (\(\text{L},\text{L}\))-\(\beta\)-hydroxybutyrate | 684 \(\pm\) 119 | 1662 \(\pm\) 382 | \(>25,000\) |
| Lithium acetacetate              | \(>25,000\)  | \(>25,000\)  | \(>25,000\) |
| Acetone                         | Inactive     | Inactive      | Inactive    |
| Sodium (\(\text{L},\text{L}\))-\(\alpha\)-hydroxybutyrate | \(>10,000\) | Inactive      | \(>10,000\) |
| Sodium lactate                  | \(>10,000\)  | Inactive      | Inactive    |
| Sodium acetate (C2)             | \(>10,000\)  | \(>10,000\)  | Inactive    |
| Sodium propionate (C3)          | \(>10,000\)  | \(>10,000\)  | Inactive    |
| Sodium butyrate (C4)            | 702 \(\pm\) 108  | 1590 \(\pm\) 211 | Inactive    |
| Pentanoic acid (C5)             | 166 \(\pm\) 33  | 402 \(\pm\) 51 | \(>10,000\) |
| Sodium hexanoate (C6)           | 133 \(\pm\) 15  | 451 \(\pm\) 91 | 995 \(\pm\) 246 |
| Heptanoic acid (C7)             | 720 \(\pm\) 132 | 2066 \(\pm\) 300 | 120 \(\pm\) 23 |
| Sodium octanoate (C8)           | 288 \(\pm\) 40  | 755 \(\pm\) 82 | 73 \(\pm\) 12 |
| Sodium deconeate (C10)          | \(>10,000\)  | \(>10,000\)  | \(>10,000\) |
| Oleic acid (C18:1)              | Inactive      | Inactive      | Inactive    |
| Linoleic acid (C18:2)           | Inactive      | Inactive      | Inactive    |
| Linolenic acid (C18:3)          | Inactive      | Inactive      | Inactive    |
| Arachidonic acid (C20:4)        | Inactive      | Inactive      | Inactive    |
| Eicosapentaenoic acid (C20:5)   | Inactive      | Inactive      | Inactive    |
| Docosahexanoic acid (C22:6)     | Inactive      | Inactive      | Inactive    |

\(^a\) Some compounds displayed activity at the highest concentration tested but not over a full dose response from which an \(EC_{50}\) could be determined. Therefore, the \(EC_{50}\) for these compounds is denoted as greater than the highest concentration tested.

\(^b\) Fatty acids with \(>10\) carbons were tested at \(10 \mu M\) only.

(3) Table I, with pentanoic (C5) and hexanoic (C6) acids being the most active on HM74a (C5, \(EC_{50} = 402 \pm 51 \mu M\); C6, \(EC_{50} = 451 \pm 91 \mu M\)) and PUMA-G (C5, \(EC_{50} = 166 \pm 33 \mu M\); C6, \(EC_{50} = 133 \pm 15 \mu M\)). HM74 has affinity for slightly longer chain length fatty acids, with a peak of activity centered on octanoic acid (C8, \(EC_{50} = 73 \pm 12 \mu M\); Table I). However, it is unlikely that these small fatty acids reach sufficient concentrations in serum to activate these receptors (19), and so their low affinity for PUMA-G/HM74a and HM74 is probably not physiologically relevant. Consistent with this, while both HM74a/PUMA-G and GPR41 have similar chain length preferences, the latter receptor has at least 10-fold higher affinity for these acids (Table I and Ref. 20). We note as well that in another study racemic (\(\text{D},\text{L}\))-\(\beta\)-OHB was not a GPR41 agonist at concentrations up to 10 mM (20). Similarly, another study found \(\text{L},\text{OH}-\text{butyrate}\) (the exact molecular structure of which was not described) to be only a very weak \(EC_{50} \approx 5 \text{mM}\) ligand of human GPR41 and GPR43, another receptor for short-chain fatty acids expressed predominantly in leukocytes (19). Finally, \(\beta\)-OHB was not an agonist of GPR81 (22), the next most phylogenetically related receptor to HM74a/PUMA-G (data not shown).

We employed an equilibrium \(^{[3]}\)Hnicotinic acid binding competition assay to ask whether nicotinic acid and \(\beta\)-OHB compete for the same binding site on the receptor. \(^{[3]}\)Hnicotinic acid bound specifically and saturably to membranes from cells expressing HM74a, but not HM74 (data not shown), with a calculated \(K_d\) of 105 \(\pm\) 9 nM (data not shown), a value in good agreement with published results (3, 4). Homologous competition with unlabeled nicotinic acid yielded a \(K_a\) of 130 \(\pm\) 14 nM, similar to the observed \(K_d\) (Fig. 1C). As in the \(^{[35]}\)GS\(\gamma\)S binding assay, AcAc displayed low apparent affinity for HM74a, but (\(\text{D},\text{D}\))-\(\beta\)-OHB bound the receptor with physiologically relevant affinity (\(K_d = 0.7 \pm 0.06 \mu M\); Fig. 1C). Similar results were observed for PUMA-G (\(K_d = 0.7 \text{mM} \pm 0.1 \text{mM}\); data not shown). Taken together, these data show that (\(\text{D},\text{D}\))-\(\beta\)-OHB is a HM74a/PUMA-G agonist and that the serum concentrations of this ketone body observed as early as 2–3 days into a fast in humans (7, 8) will result in significant receptor occupancy and activity.

Previous studies with isolated adipocytes from knock-out mice demonstrated that PUMA-G mediates the anti-lipolytic effect of nicotinic acid (3); we performed analogous experiments with (\(\text{D},\text{D}\))-\(\beta\)-OHB. Both nicotinic acid and sodium (\(\text{D},\text{D}\))-\(\beta\)-OHB suppressed free fatty acid efflux from isoproterenol-stimulated primary adipocytes from wild-type mice, the latter at concentrations consistent with the affinity determined previously (approximate \(EC_{50}\) for (\(\text{D},\text{D}\))-\(\beta\)-OHB-mediated lipolysis inhibition \(\approx 2 \text{mM}\); Fig. 2A). This suppression was PUMA-G-mediated, as nicotinic acid and (\(\text{D},\text{D}\))-\(\beta\)-OHB were without significant effect in adipocytes from PUMA-G knock-out mice (Fig. 2B). Adipocytes from PUMA-G knock-out mice were not merely refractory to lipolysis inhibition per se, as ADP inhibited fatty acid efflux equally well in wild-type and knock-out cells (Fig. 2B). The adenosine analogue phenylisopropyladensine also inhibited lipolysis equally well in wild-type and knock-out cells (data not shown). We note that the rate of both basal and isoproterenol-stimulated lipolysis was routinely lower in adipocytes from knock-out mice compared with those from wild-type animals (Fig. 2B; compare “No stimulation” and “Iso”). It is unlikely that this difference is due to a deficiency of lipid in the knock-out adipocytes, as wild-type and knock-out mice had approximately the same overall size as determined by microscopy (data not shown), and wild-type and knock-out mice had the same overall fat mass as determined by NMR spectroscopy.\(^2\)

DISCUSSION

In this work we have shown that the ketone body (\(\text{D},\text{D}\))-\(\beta\)-OHB specifically binds to and activates the adipocyte-expressed GPCRs HM74a/PUMA-G with an affinity that is well within the range of serum concentrations observed for this metabolite after \(\approx 2–3\) days of starvation in humans and \(\approx 1–2\) days in mice (7, 10). The effect of (\(\text{D},\text{D}\))-\(\beta\)-OHB is, like nicotinic acid,
lutes its own production by decreasing the serum level of fatty acid precursors available for hepatic ketogenesis. Indeed, in a study of the serum NEFA-lowering effect of β-OHB infused into humans, Senior and Loridan (9) proposed that during starvation ketone bodies exert “a fine regulatory adjustment” of their own synthesis by inhibiting adipocyte lipolysis. Such a mechanism would potentially conserve fat stores during extended starvation and attenuate excessive formation of ketoads from unrestrained lipolysis and ketogenesis. This model implies that PUMA-G knock-out mice would experience higher rates of lipolysis and ketogenesis during a fast, as (β)-β-OHB does not inhibit lipolysis in the absence of this receptor. In preliminary experiments, we have not observed significant reproducible differences between wild-type and PUMA-G knock-out animals in rate of body fat depletion or serum levels of NEFA or (β)-β-OHB during 24 and 48 h of fasting. However, we note that adipocytes from knock-out animals are refractory to stimulation with isoproterenol (Fig. 2B), perhaps suggesting a fundamental difference in lipolysis regulation in the knock-out cells that may compensate for lack of PUMA-G. Studies with inducible knock-outs of PUMA-G in adult animals may shed light on mechanisms that may compensate for lack of PUMA-G. Studies with inducible knockout of PUMA-G in adult animals may shed light on this question.

Acknowledgments—We thank Steve Colletti of Merck Research Laboratories for synthesis of lithium (DL)-β-OHB; Graeme Semple and colleagues at Arena Pharmaceuticals for synthesis of Antfarone; Sterin Tunaru of the University of Heidelberg for assistance with PUMA-G knock-out mice experiments; Donald Marsh, Kimberly Cox-York, and Marc Reitman for fasting studies with knock-out animals and advice; and Ismail Kola of Merck Research Laboratories for comments on the manuscript and support of this project.

REFERENCES

1. Tavintharan, S., and Kashyap, M. L. (2001) Curr. Atheroscler. Rep. 3, 74–82
2. Carlson, L. A. (1963) Acta Med. Scand. 173, 719–722
3. Tunaru, S., Kero, J., Schaub, A., Wufka, C., Blaukat, A., Pfeffer, K., and Offermanns, S. (2003) Nat. Med. 9, 352–355
4. Wise, A., Foord, S. M., Fraser, N. J., Barnes, A. A., Kheloumbayy, N., Eilert, M., Ignar, D. M., Murdock, P. R., Steplewski, K., Green, A., Brown, A. J., Dowell, S. J., Szekeres, P. G., Hassall, D. G., Marshall, P. H., Wilson, S., and Pike, N. B. (2000) J. Biol. Chem. 275, 9869–9874
5. Soga, T., Komohara, M., Takasaki, J., Muratou, S., Saito, T., Ohishi, T., Hiyama, H., Matsu, A., Matsushime, H., and Furuchi, K. (2003) Biochem. Biophys. Res. Commun. 303, 364–369
6. Pike, N. B., and Wise, A. (2004) Curr. Opin. Investig. Drugs 5, 271–275
7. Laflè, L. (1999) Diabetes/Metabolism Res. Rev. 15, 412–426
8. Fukao, T., Lossuchar, G. D., and Mitchell, G. A. (2004) Prostaglandins Leukotrienes Essent. Fatty Acids 70, 243–251
9. Senior, B., and Loridan, L. (1968) Nature 219, 83–84
10. Cahill, G. F. (1970) N. Engl. J. Med. 282, 668–673
11. Owen, O. E., and Reichard, G. A., Jr. (1975) Isr. J. Med. Sci. 11, 560–570
12. Bates, M. W., and Linn, L. C. (1976) Am. J. Physiol. 231, 326–330
13. Bjorntorp, P., and Schersten, T. (1967) Am. J. Physiol. 212, 683–687
14. Van Hove, J. L., Grunewald, S., Jaeken, J., Demaere, P., De Clercq, P. E., Bourdoux, F., Niezen-Koning, K., Deenhe, J. F., and Leonard, J. V. (2003) Lancet 361, 1433–1435
15. Bjorntorp, P. (1966) J. Lipid Res. 7, 621–626
16. Bjorntorp, P. (1966) Metabolism 15, 191–193
17. Meta, S. H., Lopes-Curzeda, M., and van den Bergh, S. G. (1974) FEBS Lett. 47, 19–22
18. Conklin, B. H., Farfel, Z., Lustig, K. D., Julius, D., and Bourne, H. R. (1993) Nature 361, 274–276
19. Le Poul, E., Loison, C., Struyf, S., Springael, J. Y., Lannoy, V., Deobeoq, M. E., Brezillo, S., Dupreez, V., Vassart, G., Van Damme, J., Parmentier, M., and Detheux, M. (2003) J. Biol. Chem. 278, 25481–25489
20. Xiong, Y., Miyamoto, N., Shihata, K., Valasek, M. A., Foord, S. M., Wise, A., and Dowell, S. J. (2000) J. Biol. Chem. 275, 25451–25459
21. Offermanns, S. (2003) J. Biol. Chem. 278, 27530–27538
22. Lee, D. K., Nguyen, T., Lynch, K. R., Cheng, R., Vanti, W. B., Arkhitko, O., Ignar, D. M., Murdock, P. R., Steplewski, K. M., Wilson, S., Ignar, D. M., Foord, S. M., Wise, A., and Dowell, S. J. (2003) J. Biol. Chem. 278, 11312–11319
23. Rodbell, M. (1964) J. Biol. Chem. 239, 375–380

3 D. Marsh, M. Reitman, and A. Taggart, unpublished observations.