3,5-Dihydroxybenzoic Acid, a Specific Agonist for Hydroxycarboxylic Acid 1, Inhibits Lipolysis in Adipocytes

Changlu Liu, Chester Kuei, Jessica Zhu, Jingxue Yu, Li Zhang, Amy Shih, Tareneh Mirzadegan, Jonathan Shelton, Steven Sutton, Margery A. Connelly, Grace Lee, Nicholas Carruthers, Jiejun Wu, and Timothy W. Lovenberg


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ABSTRACT

Niacin raises high-density lipoprotein and lowers low-density lipoprotein through the activation of the β-hydroxybutyrate receptor hydroxycarboxylic acid 2 (HCA2) (aka GPR109a) but with an unwanted side effect of cutaneous flushing caused by vascular dilation because of the stimulation of HCA2 receptors in Langerhans cells in skin. HCA1 (aka GPR81), predominantly expressed in adipocytes, was recently identified as a receptor for lactate. Activation of HCA1 in adipocytes by lactate results in the inhibition of lipolysis, suggesting that agonists for HCA1 may be useful for the treatment of dyslipidemia. Lactate is a metabolite of glucose, suggesting that HCA1 may also be involved in the regulation of glucose metabolism. The low potency of lactate to activate HCA1, coupled with its fast turnover rate in vivo, render it an inadequate tool for studying the biological role of lactate/HCA1 in vivo. In this article, we demonstrate the identification of 3-hydroxybenzoic acid (3-HBA) as an agonist for both HCA2 and HCA1, whereas 3,5-dihydroxybenzoic acid (3,5-DHBA) is a specific agonist for only HCA1 (EC₅₀ ~150 μM). 3,5-DHBA inhibits lipolysis in wild-type mouse adipocytes but not in HCA1-deficient adipocytes. Therefore, 3,5-DHBA is a useful tool for the in vivo study of HCA1 function and offers a base for further HCA1 agonist design. Because 3-HBA and 3,5-DHBA are polyphenolic acids found in many natural products, such as fruits, berries, and coffee, it is intriguing to speculate that other heretofore undiscovered natural substances may have therapeutic benefits.

INTRODUCTION

Niacin has been used as an effective medicine for the treatment of dyslipidemia for the past 50 years (Jacobson, 2010). Niacin exerts its therapeutic benefit through activation of the β-hydroxybutyrate receptor hydroxycarboxylic acid 2 (HCA2) (aka GPR109a; Offermanns et al., 2011) in adipocytes (Tunaru et al., 2003; Wise et al., 2003). However, the major unwanted side effect of niacin is cutaneous flushing (Jacobson, 2010), which is caused by activation of HCA2 receptors in Langerhans cells in the skin (Benyó et al., 2005, 2006). More recent studies suggest the flushing effect caused by HCA2 activation is linked to the β-arrestin signaling pathway, and this part of the signaling of HCA2 may be responsible for its role in the regulation of LDL and HDL levels (Wanders and Judd, 2011), because partial agonists for HCA2 that possess the antilipolytic, but not flush-inducing, effects failed to lower LDL and raise HDL. These results also suggest that the full agonism of HCA2 is necessary to suppress LDL and raise HDL. HCA1 (aka GPR81; Offermanns et al., 2011) is a receptor that is highly homologous to the high- and low-affinity niacin receptors, HCA2 and HCA3 (aka GPR109b; Offermanns et al., 2011), which share 52% identity at the amino acid level. Similar to HCA2, HCA1 is also highly expressed in fat tissue (Ge et al., 2008; Liu et al., 2009). Unlike HCA2, HCA1 was not found to be expressed in Langerhans cells or other immune cells in the skin. HCA1 has been identified to be an endogenous receptor for lactate (Cai et al., 2008; Liu et al., 2009). Lactate activates HCA1 and inhibits lipolysis in human, rat, and mouse adipocytes at physiologically relevant lactate concentrations (1–20 mM) but not in HCA1-deficient mouse adipocytes (Liu et al., 2009), suggesting that HCA1 contains supplemental material.

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The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: HCA, hydroxycarboxylic acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; GPCR, G protein-coupled receptor; 2-HBA, 2-hydroxybenzoic acid; 3-HBA, 3-hydroxybenzoic acid; 4-HBA, 4-hydroxybenzoic acid; 2,3-DHBA, 2,3-dihydroxybenzoic acid; 3,4-DHBA, 3,4-dihydroxybenzoic acid; 3,5-DHBA, 3,5-dihydroxybenzoic acid; 3,4,5-THBA, 3,4,5-trihydroxybenzoic acid; FFA, free fatty acid; FFAR, FFA receptor; GTPγS, guanosine 5’-O-(3-thio)triphosphate; TM, transmembrane; ECL, extracellular loop; GHB, γ-hydroxybutyrate; KO, knockout; WT, wild type.
could be a new target for dyslipidemia treatment without the unwanted side effect of cutaneous flushing. As a metabolite of glucose, lactate concentrations rise in vivo after a glucose load (DiGirolamo et al., 1992; Liu et al., 2009), and thus lactate/HCA1 may also serve a regulatory role for glucose metabolism. It has been suggested that lactate plays a role in insulin signaling, particularly in insulin-mediated antilipolytic effects (Ahmed et al., 2010). It has also been suggested that HCA1 may play a role in muscle glucose and fatty acid metabolism (Rooney and Trayhurn, 2011).

Because of the low potency of lactate as a ligand for HCA1, the high endogenous lactate concentration (>1 mM), and the fast metabolic turnover of lactate, it is difficult to pharmacologically study the in vivo function of the lactate/HCA1 system by using exogenous lactate administration. Therefore, the identification of high-affinity, nonlactate agonists and antagonists for HCA1 will greatly facilitate the in vivo study of HCA1 function. Recent mutagenesis and modeling studies suggest the carboxyl group and the hydroxyl group of lactate are responsible in part for HCA1 activation (Kuei et al., 2011). In this article, we show that by screening hydroxylated carboxylic acids, we identified 3-hydroxybenzoic acid (3-HBA) as an agonist for both HCA2 and HCA1 and 3,5-dihydroxybenzoic acid (3,5-DHBA) as a specific agonist for HCA1 but not HCA2. These two agonist tools should greatly allow the pharmacological study of the roles for HCA1 and HCA2 in the regulation of metabolism.

Materials and Methods

All compounds, except where specified, were purchased from Sigma-Aldrich (St. Louis, MO).

Recombinant Expression of HCA2, HCA3, FFAR2, FFAR3, and HCA1. The complete coding regions of human HCA2 (Genbank accession no. NY14884.1), HCA3 (Genbank accession no. EU293604.1), FFAR2 (aka GPR41) (Genbank accession no. NM_005304.3), FFAR3 (aka GPR43) (Genbank accession no. NM_005306.2), and HCA1 (Genbank accession no. EU809458) and mouse HCA2 (Genbank accession no. NM_030701.3), FFAR2 (aka GPR41) (Genbank accession no. NM_00103316.2), and HCA1 (Genbank accession no. EU809460) were cloned into a mammalian expression vector pcIneo (Promega, Madison, WI). All insert regions were sequenced to confirm the sequence identities. The receptor expression constructs were transfected into COS-7 cells by cotransfection with a human Go2 gene cloned into pcDNA3.1 (Kuei et al., 2011) by using Lipofectamine (Invitrogen, Carlsbad, CA) as the transfection reagent. Two days after transfection, the cells were harvested, and the cell pellets were used for GTPγS binding assay.

GTPγS Binding Assay. GTPγS binding assays were performed as described previously (Liu et al., 2003). In brief, cell pellets kept at −80°C were homogenized in cold Tris-HCl (10 mM, pH 7.4) plus 5 mM EDTA and centrifuged at 4°C and 400g for 10 min. The supernatants were then centrifuged at 15,000g and 4°C for 30 min. The pellets were rehomogenized in GTPγS binding buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM MgCl2, 100 mM NaCl, 10 μM GDP, and 0.5% fatty acid-free bovine serum albumin). Ligands, dissolved in water and adjusted pH to neutral, were diluted in water at various concentrations and then incubated with different receptor membranes in 96-well plates at room temperature for 20 min. [35S]GTPγS (PerkinElmer Life and Analytical Sciences, Waltham, MA) was added to each well at a final concentration of 1 nM. The final volume for each well was kept at 200 μl. The reactions were allowed to incubate at room temperature for 1 h, and the reaction mixtures were then filtered through 96-well GFC filter plates (PerkinElmer Life and Analytical Sciences) using a 96-well cell harvester (PerkinElmer Life and Analytical Sciences). The plates were then washed by using ice-cold washing buffer (10 mM Tris-HCl, pH 7.5, and 10 mM MgCl2) and counted in a micro-scintillation counter (Topcount NTX; PerkinElmer Life and Analytical Sciences). The results were analyzed by using Prism software (GraphPad Software, Inc., San Diego, CA).

Inhibition of cAMP Accumulation Assay. SK-N-MC cells stably expressing HCA1 (Liu et al., 2009) or control SK-N-MC cells were detached from culturing plates by using phosphate-buffered saline plus 10 mM EDTA and washed by using serum-free, phenol red-free Dulbecco’s modified Eagle’s medium/F12 medium (Invitrogen). Cells were then resuspended in serum-free, phenol red-free Dulbecco’s modified Eagle’s medium/F12 medium plus 2 mM 3-isobuty1-1-methylxanthine and seeded in 96-well plates at a density of 2 × 104 cells/well. Ligands of various concentrations were added to cells and incubated for 5 min. Forskolin was then added to each well (at a final concentration of 5 μM in a final volume of 200 μl) to stimulate cAMP synthesis. The reactions were allowed to incubate at room temperature for 20 min. and then stopped by adding HCl to a final concentration of 50 mM. cAMP was then extracted by incubating the plates at 4°C for 2 h, and cAMP concentrations were then measured by using cAMP FLASH plates (PerkinElmer Life and Analytical Sciences).

Lipolysis Assay. For lipolysis studies using mouse adipocytes, mature subcutaneous adipocytes were isolated from male wild-type (WT) or HCA1-deficient mice as described previously (Liu et al., 2009). Lipolysis assays were performed as described previously (Liu et al., 2009) either with or without HCA1 ligands. For studies using human cells, primary human subcutaneous adipocytes differentiated in vitro were purchased from Zen-Bio (Research Triangle Park, NC), and lipolysis studies were performed as described previously (Liu et al., 2009). Glycerol and free fatty acid (FFA) released from adipocytes were measured by using a glycerol kit (Sigma-Aldrich) and a free fatty acid measuring kit (Zen-Bio, respectively).

Molecular Modeling of HCA1. 3-HBA and 3,5-DHBA were manually docked into a previously modeled HCA1 structure (Kuei et al., 2011). The structure was then energy-minimized by using the Smart Minimizer algorithm with Generalized Born with Implicit Membrane solvent model using the CHARMMm forcefield as implemented in Discovery Studio 3.0 (Accelrys, San Diego, CA).

Results

Identification of 3-Dihydroxybenzoic Acid as an Agonist for HCA1 and HCA2. Previously, we demonstrated that, in addition to lactate, glycolate and α-hydroxybutyrate were agonists for HCA1, but with low potency (EC50 = 5–10 mM). Our recent mutation and modeling studies for HCA1 suggest that both the carboxyl and hydroxyl groups of lactate are involved in the interaction with HCA1 (Kuei et al., 2011). To identify agonists for HCA1, we tested many small organic carboxylic acids with hydroxyl groups. Among them there were 2-hydroxybenzoic acid (2-HBA), 3-HBA, and 4-hydroxybenzoic acid (4-HBA) (Fig. 1A). Our results showed that 3-HBA stimulates [35S]GTPγS binding in HCA1-expressing cell membranes (Fig. 1B) with an EC50 value of 185 ± 24 μM, but not in the control cells. 2-HBA and 4-HBA demonstrated no detectable agonist activity for HCA1. Counter-screening using membranes from HCA2-, HCA3-, or FFAR3-expressing cells indicated that whereas 2-HBA, 3-HBA, and 4-HBA activate HCA2 (Fig. 1C) with various potencies and efficacies, none of them activate HCA3 (Fig. 1D) or the propionate receptor FFAR3 (Fig. 1E). In addition, these compounds were tested as agonists for the acetate receptor FFAR2 and histamine.
mine H₃ receptor and showed no agonist activity for either receptor (data not shown).

3,5-Dihydroxybenzoic Acid Specifically Activates HCA1. We next tested the derivatives of 3-HBA (Fig. 2A) including 2,3-dihydroxybenzoic acid (2,3-DHBA), 3,4-dihydroxybenzoic acid (3,4-DHBA), 3,5-DHBA, and 3,4,5-trihydroxybenzoic acid (3,4,5-THBA; aka gallic acid) as potential agonists for HCA1. The results showed that 3,5-DHBA activates HCA1 (EC₅₀/H₁₁₀₀₅₁₉₁/H₁₁₀₀₆₂₆/H₉₂₆₂ M) with an Eₘ₅ value comparable with that of L-lactate. However, 2,3-DHBA, 3,4-DHBA, and 3,4,5-THBA showed no agonist activity (Fig. 2B). Counter-screening of 3,5-DHBA against HCA2, HCA3, FFAR3, and FFAR2 demonstrated that 3,5-DHBA is a very selective agonist for HCA1. 3,5-DHBA only showed marginal agonist activity for HCA2 at concentrations more than 10 mM (Fig. 2C) and demonstrated no activity for HCA3 (Fig. 2D), FFAR3 (Fig. 2E), or FFAR2 (data not shown).

Fig. 1. Identification of 3-HBA as an agonist for human HCA1 and HCA2. A, structures of niacin, 2-HBA, 3-HBA, and 4-HBA are shown. B to E, niacin and the hydroxybenzoic acids were tested for their agonistic activity for human HCA1 (B), HCA2 (C), HCA3 (D), and FFAR3 (E). Lactate, niacin, aci-fran, and propionate were used as positive controls for human HCA1, HCA2, HCA3, and FFAR3, respectively. GTPγS binding assay was used to characterize receptor activation. [³⁵S]GTPγS binding with ligand stimulation is expressed as percentage of basal incorporation without ligand stimulation. Each data point was assayed in triplicate, and means ± S.E.M. are shown.

Modeling of 3,5-DHBA and HCA1 Interactions. Previous modeling of the HCA1 and lactate interaction (Kuei et al., 2011) suggests that the carboxyl group of lactate interacts with Arg99 (at TM3) and Arg240 (at TM6) of HCA1, whereas the hydroxyl group of lactate interacts with Arg71 (at TM2) and Glu166 (at the extracellular loop, ECL2) of HCA1. In this study, we attempted to model both 3-HBA and 3,5-DHBA with HCA1 to look for similar interactions. The results show that whereas the carboxyl group of 3-HBA interacts with Arg99 (at TM3) and Arg240 (at TM6) of HCA1, the 3-hydroxyl group interacts with Glu166 via hydrogen bonding. Likewise, for 3,5-DHBA, the carboxyl group interacts with Arg99 as well as Arg240 of HCA1 and the 3-hydroxyl group interacts with Glu166. In addition, the 5-hydroxyl group of 3,5-DHBA is able to interact with Arg71 (at TM2) through hydrogen bonding (Fig. 3, A and B). For HCA2 and HCA3, residues Arg111 and Arg251 corresponding to
Arg99 and Arg240 in HCA1 are conserved. However, at ECL2, corresponding to Glu166 of HCA1, it is Ser178 in HCA2, which is able to interact with the 3-hydroxyl group of 3-HBA through hydrogen bonding. In contrast, HCA3 has Ile178 at this position (Kuei et al., 2011). The hydrophobic residue Ile178 at this position clashes with the 3-hydroxyl
group of 3-HBA and therefore prevents the binding/activation of HCA3 by 3-HBA. In TM2, corresponding to Arg71 of HCA1, it is a hydrophobic residue, Leu83, for HCA2 (and Val for HCA3) (Fig. 3C) that forms a static clash with the 5-hydroxyl group of 3,5-DHBA, therefore preventing the interactions between 3,5-DHBA and HCA2 (and HCA3).

3-HBA and 3,5-DHBA Inhibit cAMP Accumulation in HCA1-Expressing Cells. Previous studies showed that HCA1 is coupled to G_i/o proteins and activation of HCA1 by lactate leads to the inhibition of cAMP accumulation in HCA1-expressing cells (Liu et al., 2009). In this study, we tested both 3-HBA and 3,5-DHBA, in addition to lactate, for their ability to inhibit cAMP accumulation in SK-N-MC cells stably expressing human HCA1 (Liu et al., 2009). The results showed that, like lactate, both 3-HBA and 3,5-DHBA inhibited forskolin-induced cAMP accumulation in HCA1-expressing cells (Fig. 4A) but not in control SK-N-MC cells (Fig. 4B). Compared with lactate (IC_{50} = 3.74 ± 0.62 mM), 3-HBA and 3,5-DHBA demonstrated significantly higher potency at inhibiting cAMP accumulation in HCA1-expressing cells with IC_{50} values of 81 ± 14 and 112 ± 21 μM, respectively.

Pharmacological Characterization of 3-HBA and 3,5-DHBA using Mouse Receptors. We next characterized 3-HBA and 3,5-DHBA by using recombinant mouse HCA1, HCA2 (aka PUMA-g), FFAR3, and FFAR2. The results showed that, similar to the human receptors, 3-HBA activates both mouse HCA1 (EC_{50} = 186 ± 37 μM) (Fig. 5A) and HCA2 (EC_{50} = 158 ± 23 μM) (Fig. 5B) but not mouse FFAR3.
(Fig. 5C) and mouse FFAR2 (Fig. 5D). In addition, 3,5-DHBA is a very selective agonist for mouse HCA1 (EC$_{50}$ = 172 ± 28 μM). It did not activate mouse HCA2 until its concentration reached 10 mM. At 10 mM concentration, 3,5-DHBA stimulated marginal response in mouse HCA2-expressing cells (Fig. 5B). No stimulation of mouse FFAR3 and FFAR2 was observed at any concentration tested for 3,5-DHBA, whereas the control, propionate, activates mouse FFAR3 and FFAR2 at EC$_{50}$ values of approximately 40 μM (Fig. 5C) and 100 μM (Fig. 5D), respectively.

**3,5-DHBA Inhibits Lipolysis in Adipocytes.** Lactate has been shown to inhibit lipolysis in adipocytes (Liu et al., 2009). 3-HBA and 3,5-DHBA were tested for their activity to inhibit lipolysis in mature adipocytes isolated from mouse subcutaneous and epididymal fat. These effects were compared with those of L-lactate and propionate. Propionate was used as a control for the inhibition of lipolysis through the activation of FFAR3 and FFAR2 (Lee et al., 2008). Glycerol and free fatty acid releases were used as parameters for lipolysis. For adipocytes from subcutaneous fat, our results showed that, similar to lactate but with higher potency, both 3-HBA and 3,5-DHBA inhibited free fatty acid (Fig. 6A) and glycerol (Fig. 6B) release. The inhibitory effects of lactate and 3,5-DHBA were abolished in HCA1-deficient mouse adipocytes. The inhibition of lipolysis by 3-HBA in adipocytes from HCA1-deficient mice was only partially reduced. In contrast, the inhibitory effects of propionate were found to be at very similar levels for adipocytes isolated from either

![Free Fatty Acid Release](image)

![Glycerol Release](image)

![Free Fatty Acid/Glycerol Release](image)

**Fig. 6.** 3-HBA and 3,5-DHBA inhibits lipolysis in adipocytes. A and B, 3,5-DHBA inhibits lipolysis in adipocytes isolated from wild-type mice but not from HCA1-deficient (KO) mice. Different reagents were added to the isolated adipocytes to inhibit lipolysis. Mature subcutaneous adipocytes isolated from WT or KO mice were used in lipolysis studies. FFA (A) and glycerol (B) releases were measured from the adipocyte incubation buffer after 2 h of incubation. FFA release (314 μM for the WT and 257 μM for KO) and the glycerol release (162 μM for WT and 137 μM for KO) from the control group were set as 100%, and results from other groups are presented as percentage of the respective controls. Propionate was used as a positive control for the inhibition of lipolysis in both WT and KO cells. C, 3-HBA and 3,5-DHBA inhibit lipolysis in human subcutaneous adipocytes. Human subcutaneous adipocytes differentiated in 24-well culture plates were used. FFA release (153 μM) and glycerol release (76 μM) from the control group were set as 100%, and results from other groups are presented as percentage of the respective controls. Each data point was assayed in triplicate, and means ± S.E.M. are shown. Lipolysis buffer was served as the control, and NaCl solution (25 mM) was used as an additional control for comparison with lactate in all lipolysis assays.
wild-type or HCA1-deficient mice. Very similar results have been observed when mature adipocytes were isolated from epididymal fat and tested for lipolysis under similar conditions (data not shown). 3-HBA and 3,5-DHBA were also tested in human subcutaneous adipocytes differentiated in vitro, and both of them demonstrated strong inhibitory effects on lipolysis (Fig. 6C).

Discussion

Identification of 3,5-DHBA as a Potential Tool for HCA1 In Vivo Studies. In this article, we first described the finding of 3-HBA and 3,5-DHBA as nonlactate HCA1 agonists with much improved potency (100–200 μM levels versus 5 mM for l-lactate). In addition, we identified 3,5-DHBA as a specific agonist for the HCA1 receptor. The activation of HCA1 was demonstrated by ligand-stimulated GTPγS binding with HCA1 coexpressed with Go2, and very similar results were observed (Supplemental Fig. 1). The reason we choose Go2 for coexpression with HCA1 for in vitro studies is because in our hands, many Gt-coupled receptors, when coexpressed with Go2, produce better signal/noise ratio in GTPγS binding assay. L-Lactate is an energy source, a metabolite, as well as a signaling molecule. In vivo, l-lactate can be rapidly converted into pyruvate, which either enters the tricarboxylic acid cycle to generate energy or is converted into glucose through gluconeogenesis. Therefore, studying the in vivo function of HCA1 via lactate administration will be hampered by the fast metabolic turnover of lactate. In addition, because lactate is a very-low-affinity ligand, excessive amounts of lactate have to be administered to achieve pharmacological activity, which further confounds the interpretation of the data. The identification of 3,5-DHBA as a specific agonist for HCA1 will provide a potential tool for HCA1 activation, avoiding some of the challenges presented by lactate administration. In addition, 3-HBA and 3,5-HBA are natural products belonging to the class of molecules generally considered as safe. Because such substances can often be administered in animals (even humans) at very high doses without significant adverse effects, these compounds may be useful tools for studying HCA1 and HCA2 functions. Therefore, the finding of 3,5-DHBA as a specific higher-affinity agonist for HCA1 provides a potential tool for the in vivo study of HCA1, which will further extend our knowledge of the role of this receptor in human physiology. Because recent studies showed that HCA2 partial agonists failed to lower LDL and raise HDL, it will be interesting to see what HCA1 full agonists can do in lowering LDL and raising HDL.

The Structure/Activity Relationship of HCA1 Ligands Sheds Light for Future HCA1 Ligand Design. HCA1, HCA2, and HCA3 are three closely related GPCRs that are highly expressed in adipocytes and possess antilipolytic effects. Although lactate is probably the endogenous ligand for HCA1, β-hydroxybutyrate and 3-OH-oc-
ganoic acid are proposed as the endogenous ligands for HCA2 (Taggart et al., 2005) and HCA3 (Ahmed et al., 2009), respectively. Molecular modeling of HCA2 (Tunaru et al., 2005) and HCA1 (Kuei et al., 2011) indicate that both the carboxyl and the hydroxyl groups are very critical for receptor activation, and the position of the hydroxyl group is very critical for receptor selection. For instance, when the hydroxyl group is at the 2-position (lactate and α-hydroxybutyrate), these organic acids can activate HCA1 but not HCA2 and HCA3. In contrast, when the hydroxyl group is at the 3-position (β-hydroxybutyrate and 3-OH-octanoic acid), these organic acids activate HCA2 or HCA3 but not HCA1. In our previous report (Liu et al., 2009), we also showed that γ-hydroxybutyrate (GHB) can activate HCA1 with full efficacy, albeit with a very low potency (EC50 = 15 mM). In this article, we show that 3-HBA activates HCA1 with much improved potency. Structural comparison between GHB and 3-HBA shows that the tetra lengths of the hydroxyl group to the carboxyl group in the two molecules are the same. Although the hydroxyl group in GHB has great flexibility (as does the hydroxyl group in lactate), 3-HBA has a much more rigid structure, and the flexibility of the hydroxyl group is restrained. It is possible that this increased rigidity is responsible for the increased potency to HCA1. It is intriguing to speculate whether further increasing the restraint of the hydroxyl group at the 3-position of 3-HBA by chemical modification will lead to improved potency.

To elucidate the physiological function of HCA1, pharmacological tools are required that can elicit the specific activation of HCA1 in vivo. Novel, nonlactate-like HCA1 ligands will greatly facilitate the research. In this article, we show that 3,5-DHBA is a very selective ligand for HCA1 over its closely related GPCRs such as HCA2 and HCA3. The addition of the 5-hydroxyl group is the key leading to the specificity, compared with having only the 3-hydroxy benzoic acids, which are not selective for HCA1 over HCA2. Molecular modeling suggests that the 5-hydroxyl group probably interacts with Arg71 in the TM2 through hydrogen bonding. HCA1 and HCA2 are very closely related; however, at TM2, the corresponding residue is a Leu in HCA2 and a Val in HCA3, which are hydrophobic residues and clash with the addition of the hydrophilic 5-hydroxyl group of 3,5-DHBA, providing an explanation for why 3,5-DHBA activates HCA1 but not HCA2 or HCA3. These results also suggest that, for future computer-aided drug design, Arg71 in TM2 should be one of the focal points for targeting HCA1 specificity.

Are Lactate, 3-HBA, and 3,5-DHBA Allosteric or Orthosteric Ligands for HCA1? Lactate has an EC50 value of approximately 5 mM for HCA1. 3-HBA and 3,5-DHBA, although with much improved potency (EC50 ~150 μM), still have relatively low potency compared with other classes of GPCR agonists. This leads to the question of whether lactate, 3-HBA, and 3,5-DHBA are allosteric ligands for HCA1. In a previous publication, we demonstrated that lactate was capable of inducing receptor internalization, a phenomenon associated with orthosteric receptor activation (Liu et al., 2009; Kuei et al., 2011). We also proposed a model for how lactate might fit into the putative binding pocket, which is analogous to many of the biogenic amine receptors. The model suggests that both the carboxyl group and the hydroxyl group of lactate interact with HCA1. In this article, for 3-HBA and 3,5-DHBA both carboxyl and hydroxyl groups are essential for HCA1 activation, suggesting that these HBA ligands interact with the same site as lactate. The molecular modeling supports this hypothesis showing that whereas the carboxyl group interacts with Arg99 and Arg240 of HCA1,
the 3-hydroxyl group interacts with Glu166 at the ECL2, and the 5-hydroxyl group interacts with Arg71 at the TM2 of HCA1, which are the same sites we proposed with which lactate interacts. Additional evidence using a combination of lactate and 3-HBA or 3,5-DHBA showed that at low concentrations lactate and 3-HBA or 3,5-DHBA have additive effects on HCA1 activation, but there is no synergistic effect at high concentrations (Supplemental Fig. 2). The combination of lactate and 3-HBA or 3,5-DHBA did not change the E_{	ext{max}} value, further indicating that 3-HBA or 3,5-DHBA interacts with HCA1 at the same site as lactate. However, compared with lactate, 3-HBA and 3,5-DHBA have much more rigid structures, allowing them to bind HCA1 with higher affinities.

Can 3-HBA and 3,5-HBA in Food Products Have Pharmacological Activities? Fruits and whole-grain foods have been considered as healthy foods. They are rich in phenolic acids and alkylresorcinols. Phenolic acids are a class of organic molecules with a phenolic ring and an organic carboxylic acid group. They are rich in chocolates, coffee, fruits, vegetables, red wines, and green teas and have been suggested to play regulatory roles in cholesterol metabolism (Uto-Kondo et al., 2010), antibiostasis (Hsu et al., 2006), glucose metabolism (Jung et al., 2007), and immune functions (Hole et al., 2009). Hydroxybenzoic acids, including 3-HBA and 3,5-DHBA, belong to the subfamily of hydroxylated phenolic acids and have been found to be rich in green tea (Gruz et al., 2008), grapefruit, and olive oil (Khadem and Marles, 2010). 3,5-DHBA is also identified in human urine, which is suggested as a metabolite of alkylresorcinols from foods such as whole-grain wheat/cereals (Aubertin-Leheudre et al., 2008), grapefruit, and olive oil (Khadem and Marles, 2010). The identification of 3-HBA as an agonist for HCA1 and HCA2 and 3,5-DHBA as a specific agonist for HCA1 led us to speculate whether 3-HBA and 3,5-DHBA in the food substances may be having direct pharmacological effects on HCA receptors. Because of their relatively low potency, one obvious question will be whether the amount of 3-HBA and 3,5-DHBA exposure from the food consumption can reach effective levels. Although the concentration of 3-HBA and 3,5-DHBA absorbed from food may be much lower than their EC_{50} values, we should not rule out their potential effects. The average concentration of lactate in human plasma is approximately 1 mM. Additional agonism for HCA1 from natural products in food may provide added activation of HCA1 in addition to the existing agonism by lactate, which is supported by our in vitro results (Supplemental Fig. 2). Although this is currently a speculation, it is certainly plausible that natural acids in foods may have coevolved to provide metabolic signals to animals and humans. Clearly, further studies are required to investigate the role of dietary 3-HBA, 3,5-DHBA, and other organic acids in the pharmacological activation of HCA1.

Authorship Contributions

Participated in research design: Liu, Shih, Mirzadegan, Shelton, Wu, and Lovenberg.

Conducted experiments: Liu, Kuei, Zhu, Yu, Zhang, Shih, Shelton, Lee, and Wu.


Wrote or contributed to the writing of the manuscript: Liu, Kuei, Shih, Mirzadegan, Connelly, Carruthers, Wu, and Lovenberg.

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