20-HETE Signals Through G-Protein–Coupled Receptor GPR75 (G\textsubscript{q}) to Affect Vascular Function and Trigger Hypertension


Rationale: 20-Hydroxyeicosatetraenoic acid (20-HETE), one of the principle cytochrome P450 eicosanoids, is a potent vasoactive lipid whose vascular effects include stimulation of smooth muscle contractility, migration, and proliferation, as well as endothelial cell dysfunction and inflammation. Increased levels of 20-HETE in experimental animals and in humans are associated with hypertension, stroke, myocardial infarction, and vascular diseases.

Objective: To date, a receptor/binding site for 20-HETE has been implicated based on the use of specific agonists and antagonists. The present study was undertaken to identify a receptor to which 20-HETE binds and through which it activates a signaling cascade that culminates in many of the functional outcomes attributed to 20-HETE in vitro and in vivo.

Methods and Results: Using crosslinking analogs, click chemistry, binding assays, and functional assays, we identified G-protein receptor 75 (GPR75), currently an orphan G-protein–coupled receptor (GPCR), as a specific target of 20-HETE. In cultured human endothelial cells, 20-HETE binding to GPR75 stimulated G\textsubscript{q11} protein dissociation and increased inositol phosphate accumulation and GPCR-kinase interacting protein-1-GPR75 binding, which further facilitated the c-Src–mediated transactivation of epidermal growth factor receptor. This results in downstream signaling pathways that induce angiotensin-converting enzyme expression and endothelial dysfunction. Knockdown of GPR75 or GPCR-kinase interacting protein-1 prevented 20-HETE–mediated endothelial growth factor receptor phosphorylation and angiotensin-converting enzyme induction. In vascular smooth muscle cells, GPR75–20-HETE pairing is associated with G\textsubscript{q11} and GPCR-kinase interacting protein-1–mediated protein kinase C–stimulated phosphorylation of MaxiK\textsubscript{\beta}, linking GPR75 activation to 20-HETE–mediated vasoconstriction. GPR75 knockdown in a mouse model of 20-HETE–dependent hypertension prevented blood pressure elevation and 20-HETE–mediated increases in angiotensin-converting enzyme expression, endothelial dysfunction, smooth muscle contractility, and vascular remodeling.

Conclusions: This is the first report to identify a GPCR target for an eicosanoid of this class. The discovery of 20-HETE–GPR75 pairing presented here provides the molecular basis for the signaling and pathophysiological functions mediated by 20-HETE in hypertension and cardiovascular diseases. (Circ Res. 2017;120:1776-1788. DOI: 10.1161/CIRCRESAHA.116.310525.)

Key Words: cardiovascular diseases ■ cytochrome P-450 enzyme system ■ hypertension ■ receptor, epidermal growth factor ■ vascular remodeling

Hypertension is the leading cause of stroke and cardiovascular diseases and the chief risk factor for global disease burden. With few exceptions, the molecular bases of the most common forms of human hypertension are yet to be defined and, thus, its early diagnosis and clinical management remains challenging, reflecting the complexity of a disease in which multiple environmental and genetic factors contribute to its multifaceted cause. Studies from us and others added 20-hydroxyeicosatetraenoic acid

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Novelty and Significance

What Is Known?

- 20-hydroxyeicosatetraenoic acid (20-HETE), a bioactive lipid autacoid, plays a role in the pathogenesis of hypertension, stroke, myocardial infarction, renal failure, and diabetes mellitus.
- The actions of 20-HETE seem to involve interaction with a specific receptor/target molecule; yet, the cellular presence of a specific receptor for 20-HETE has not been demonstrated.

What New Information Does This Article Contribute?

- This study identifies G-protein receptor 75 (GPR75), an orphan G-protein–coupled receptor, as the putative 20-HETE-receptor. This is the first time a G-protein–coupled receptor has been identified for an eicosanoid of this class.
- The experimental approach to identifying a specific receptor target for 20-HETE included the use of a click chemistry crosslinking analog, proteomics, protein partner analysis, receptor binding assays, gene silencing, and functional assays.
- 20-HETE binding to GPR75 activates distinct signaling cascades in endothelial and vascular smooth muscle cells that culminate in the activation of vascular angiotensin-converting enzyme expression, endothelial dysfunction, contractility, remodeling, and hypertension.
- The discovery of 20-HETE–GPR75 pairing opens the door for future research with regard to how this lipid–receptor interaction is involved in a variety of pathologies known to be closely mediated and associated with 20-HETE.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>20-HETE</td>
<td>20-hydroxyeicosatetraenoic acid</td>
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<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>GIT1</td>
<td>G-protein–coupled receptor kinase interactor 1</td>
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<td>GPCR</td>
<td>G-protein–coupled receptor</td>
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<td>GPR75</td>
<td>G-protein receptor 75</td>
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<tr>
<td>HIC-5</td>
<td>hydrogen peroxide–inducible clone 5</td>
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<tr>
<td>IP-1</td>
<td>inositol phosphate</td>
</tr>
<tr>
<td>MaxiKβ</td>
<td>large conductance voltage and calcium-activated potassium subunit β</td>
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<td>NO</td>
<td>nitric oxide</td>
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The proposed contributions of 20-HETE to the cause of cardiovascular diseases stem primarily from its reported actions on the vasculature. 20-HETE is a potent vasoactive eicosanoid whose vascular effects include stimulation of smooth muscle contractility, migration, and proliferation, as well as endothelial cell (EC) dysfunction and inflammation. While studying 20-HETE triggered signaling cascades mediating endothelial dysfunction and activation, we identified the phosphorylation of the epidermal growth factor receptor (EGFR) as a first step in a mitogen-activated protein kinase-inhibitor of nuclear factor κ-B kinase subunit β-nuclear factor κB transduction pathway leading to endothelial nitric oxide (NO) synthase uncoupling, inflammatory cytokine production, and to increases in angiotensin-converting enzyme (ACE) expression and activity. Although the mechanisms by which 20-HETE activates EGFR phosphorylation remained unknown, we postulated that 20-HETE could either (1) cross the cell membrane and stimulate tyrosine kinases within the intracellular milieu or (2) interact with a G-protein–coupled receptor (GPCR), the activation of which could lead to transactivation of the EGFR, as was documented for many autacoids including angiotensin II. Interestingly, Akbulut et al showed that 20-HETE activates the Raf/MEK/ERK pathway in renal epithelial cells through an EGFR- and c-Src–dependent mechanism and proposed the presence of a 20-HETE–specific GPCR-EGFR transactivation through c-Src. The presence of a receptor/binding site for 20-HETE was suggested by the development of specific analogs and antagonists that mimic or block many of the known 20-HETE bioactions.
chemistry, proteomics, protein partner analysis, receptor binding assays, and gene silencing, we identified G-protein receptor 75 (GPR75), currently an orphan GPCR, as the 20-HETE receptor capable of binding 20-HETE with high affinity and of activating a signaling cascade that culminates in many of the functional outcomes attributed to 20-HETE in vitro and in vivo.

Methods

Expanded Methods are presented in the Online Data Supplement. Primary (passages 2–3) human microvascular ECs (Invitrogen, Carlsbad, CA) and the rat aortic vascular smooth muscle cell line A7r5 (ATCC, Manassas, VA) were used for all in vitro experiments. Click-in chemistry and binding studies were conducted using EC membranes. Pull-down experiments and Western blot analysis of GPR75 was performed using a polyclonal antibody against the c-terminal (sc-164538; Santa Cruz, Biotechnology, Dallas, TX), which recognized a single 54- to 55-kDa protein band. All experimental protocols were approved by the Institutional Animal Care and Use Committee and by the Institutional Biosafety Committee. The generation and phenotypic characterization of the Cyp4a12 transgenic mice in which the expression of the Cyp4a12-20-HETE synthase is under the control of a C57BL/6 mouse by quantitative polymerase chain reaction (qPCR) and GeneAtlas demonstrate tissue-specific expression of Gpr75 to databases including the Human Protein Atlas, BioGPS, and NCBI (National Center for Biotechnology Information) protein partner databases. The analysis revealed an association of HIC-5 and GIT1 with a candidate orphan receptor GPR75. To this end, in 1 of 3 separate experiments GPR75-specific sequences were detected with CI of 99.045%, based on peptide fingerprint mass and fragmentation mapping using the Mascot search engine, encouraging further investigation as to a potential relationship between 20-HETE and GPR75. Moreover, databases including the Human Protein Atlas, BioGPS, and GeneAtlas demonstrate tissue-specific expression of Gpr75 to be broadly distributed across various organs including the brain, endocrine tissues, lung, kidney, heart, adipose tissues, aorta, and other tissues. Our assessment of Gpr75 expression in tissues from C57BL/6 mice by quantitative polymerase chain reaction (qPCR) concurred with the distribution profile presented in these databases (Online Figure II). Many of these tissues are heavily vascularized and have the capacity to produce 20-HETE.

Results

Identification of GPR75-20-HETE Pairing

We synthesized an analog of 20-HETE, 20-azido-N-((4-(3-(4-benzoylphenyl)propanamido)-phenyl)sulfonyl)-19(S)-hydroxyecosa-5(Z),14(Z)-dienamide (Figure 1A) that contained both benzophenone, a photoreactive crosslinker for protein binding, and an azide, for selective binding and labeling to a click-chemistry dibenzocyclooctyne 800CW Infrared Dye. 20-Apheda functions as a 20-HETE antagonist as inferred from its ability to block 20-HETE–mediated sensitization of phenylephrine vasoconstriction by decreasing (4-fold) the EC50 (Figure 1B). Incubation of 10 nmol/L of 20-Apheda with membrane fractions of human EC (20 µg) followed by 15 minutes of UV (365 nm) crosslinking and 1 hour of incubation with the click reagent (dibenzocyclooctyne-IRdye 800CW, LiCor; 50 µmol/L) yielded several bands, including a dominant band located at 47 to 49 kDa (Figure 1C). This labeling profile of several bands is characteristic of many click-chemistry interactions whereby multiple proteins in close proximity to the binding site of the compound, in this case 20-Apheda, are labeled. Nevertheless, the labeling of these bands by 20-Apheda (100 µmol/L) was competed by excess amounts of 20-HETE (100 µmol/L), but not of 12-hydroxyeicosatetraenoic acid (100 µmol/L; Figure 1D and 1E).

In-gel 20-Apheda–protein complexes were extracted from independent incubations and protein identified by Applied Biomics (Hayward, CA). Protein identification of the dominant band location was based on peptide fingerprint mass mapping (using MS data) and peptide fragmentation mapping (using MS/MS data). The Mascot search engine was used to identify proteins from primary sequence databases. Analysis of sequenced samples from the dominant band location identified several proteins and domains as top hits including TGFBI11 or hydrogen peroxide–inducible clone 5 (HIC-5), POTE ankyrin family members, Zinc finger proteins, and GTP-binding proteins as top hits (Online Figure I). Further analysis also suggested domains and proteins associated with G-protein–coupled receptor kinase interactor 1 (GIT1), a GPCR-kinase–interacting protein-1 scaffold protein with ADP-ribosylating factor GTPase activity providing clues toward the possibility of a multiprotein receptor complex in close proximity to 20-Apheda’s binding site.

With the identification of these 2 particular proteins, we utilized protein partner analysis against orphan Gq Class A receptors referencing STRING (protein-protein interaction networks STRING [String-db.org]) and NCBI (National Center for Biotechnology Information) protein partner databases. The analysis revealed an association of HIC-5 and GIT1 with a candidate orphan receptor GPR75. To this end, in 1 of 3 separate experiments GPR75-specific sequences were detected with CI of 99.045%, based on peptide fingerprint mass and fragmentation mapping using the Mascot search engine, encouraging further investigation as to a potential relationship between 20-HETE and GPR75. Moreover, databases including the Human Protein Atlas, BioGPS, and GeneAtlas demonstrate tissue-specific expression of Gpr75 to be broadly distributed across various organs including the brain, endocrine tissues, lung, kidney, heart, adipose tissues, aorta, and other tissues. Our assessment of Gpr75 expression in tissues from C57BL/6 mice by quantitative polymerase chain reaction (qPCR) concurred with the distribution profile presented in these databases (Online Figure II). Many of these tissues are heavily vascularized and have the capacity to produce 20-HETE.

Analyses of competition binding studies were performed. As seen in Figure 2A, 20-HETE but not 12(S)-HETE displaced bound [3H] 20-HETE (6.67 nmol/L) from EC membranes in a concentration-dependent manner with a calculated Kd of 3.75 nmol/L (Figure 2B). Knockdown of GPR75 (Figure 2C) prevented 3H-20-HETE binding to EC membranes (Figure 2D). Likewise, addition of the 20-HETE antagonist 20-5,16-HEDGE inhibited 3H-20-HETE binding to EC membranes (Figure 2F). This experiment confirmed our initial target protein identification (Online Figure I). Furthermore, the Kd values in these experiments (Figure 2B and 2E) accurately represent 20-HETE’s biological activities wherein maximal changes in NO bioavailability and ACE expression have been seen in the range of 1 to 10 nmol/L. Because GPR75 is a Gq-coupled receptor, the ability of 20-HETE to increase inositol phosphate (IP-1) accumulation in EC as an index of its activation was further assessed. Exposure of EC to 20-HETE (10 nmol/L) for 5 minutes increased IP-1 accumulation by 4-fold; this increase was not observed in GPR75-knockdowned EC (Figure 2G and 2H). All together, these results show that 20-HETE binds to EC membranes, pairs with GPR75 in a specific manner, and functionally activates the receptor.
GPR75 Is Expressed in the Vascular Endothelium and Is Associated With \( \text{G}_\alpha_{q/11} \), GIT1, and HIC-5

Antibodies against the GPR75 C-terminal, which recognize a single 54- to 55-kDa protein band (Online Figure III), were used in all subsequent experiments. A representative image of kidney sections costained with antibodies against GPR75 and CD31, an EC marker, showed a clear colocalization of GPR75 to the vascular endothelium and expression of GPR75 can be observed throughout the vessel (Figure 3A). Moreover, immunoprecipitation and immunoblotting experiments indicated that \( \text{G}_\alpha_{q/11} \) as well as GIT1 and HIC-5 are associated with GPR75 in EC (Figure 3B).

20-HETE Alters the Association of GPR75 With \( \text{G}_\alpha_{q/11} \), GIT1, and HIC-5 in Endothelial Cells

Incubation of EC with 20-HETE (5 minutes) decreased GPR75-\( \text{G}_\alpha_{q/11} \) association by 44% and 51% at 1 and 10 nmol/L (Figure 3C). In addition, 20-HETE also increased GPR75-GIT1 binding by 2-fold and HIC-5-GPR75 dissociation by 60% (Figure 3D and 3E). Importantly, 20-6,15-HEDGE, an effective 20-HETE antagonist in vitro and in vivo, did not increase GIT1–GPR75 association. Moreover, it prevented 20-HETE–stimulated GIT1–GPR75 association (Figure 3F).

GPR75 and GIT1 Are Required for 20-HETE–Mediated EGFR Phosphorylation and Downstream Signaling

Previously, we documented that phosphorylation of EGFR is an early step in 20-HETE–mediated activation of a mitogen-activated protein kinase-inhibitor of nuclear factor \( \kappa \)-B kinase subunit \( \beta \)-nuclear factor \( \kappa \)B signaling leading to endothelial NO synthase uncoupling, ACE induction, and inflammatory cytokine production in EC. To investigate the role of GPR75 and GIT1 in 20-HETE–mediated phosphorylation of EGFR, small interfering RNAs (siRNA) against GPR75 and GIT1 were used. Transfection of EC with siRNAs against GPR75 and GIT1 produced a maximal knockdown of 85% and 70%, respectively (Figure 4A and 4B). As shown previously,
20-HETE increases total tyrosine phosphorylation of immunoprecipitated EGFR in EC transduced with control siRNA by 1.99±0.16-fold (Figure 4C). 20-HETE–stimulated EGFR tyrosine phosphorylation was completely prevented in EC transduced with either GPR75 or GIT1 siRNA (Figure 4C). In addition, within the same time frame in which 20-HETE stimulated a 2.3-fold increase in EGFR phosphorylation (Figure 4D), it also decreased the association of c-Src with GIT1 by 72% (Figure 4E) and increased association of c-Src with EGFR by 110% (Figure 4F), suggesting that 20-HETE binding to GPR75 activates a c-SRC–mediated EGFR phosphorylation via GIT1.

20-HETE–Mediated Induction of ACE mRNA Requires GPR75

One of the most prominent effects of 20-HETE in the vascular endothelium is induction of ACE transcription and activity.22,24 Here, we show that suppression of GPR75 in EC by GPR75-specific siRNAs negated the 3.4-fold induction of ACE mRNA by 20-HETE (Figure 4G), indicating that GPR75–20-HETE pairing is a necessary step for 20-HETE–mediated induction of ACE. Importantly, the chemokine CCL5, a proposed GPR75 ligand,35 did not induce ACE transcription nor did it increase EGFR tyrosine phosphorylation or Gαq/11–GPR75 association in EC (Online Figure IV).

GPR75 Knockdown Prevents 20-HETE–Dependent Hypertension, Vascular Dysfunction, and Remodeling

The Cyp4a12 transgenic mice (Cyp4a12tg) in which the expression of the Cyp4a12–20-HETE synthase is under the control of doxycycline display, on administration of doxycycline, increased levels of 20-HETE that is associated with vascular dysfunction and hypertension, both of which are...
prevented or reversed by inhibiting the biosynthesis or blocking the actions of 20-HETE. We used this model to assess whether GPR75 is necessary for the prohypertensive actions of 20-HETE. Mice were given a bolus injection of either control or GPR75-targeted shRNA lentiviral particles into the retroorbital sinus followed by administration of doxycycline in the drinking water to induce Cyp4a12–20-HETE synthase. As expected, administration of doxycycline to Cyp4a12tg mice that received a bolus of control shRNA lentiviral particles or its vehicle resulted in a rapid and sustained increase in systolic blood pressure measured by the tail cuff method (135±2 and 131±3 mm Hg, respectively; Figure 5A and 5B). In contrast, doxycycline administration to Cyp4a12tg mice that received a bolus of GPR75-targeted shRNA lentiviral particles failed to increase blood pressure (110±2 mm Hg; tail cuff, Figure 5A and 5B and telemetry; Online Figure V). Western blot analysis of renal preglomerular microvessels from mice receiving GPR75-targeted shRNA lentiviral particles confirmed an 80% knockdown of GPR75 levels (Figure 5C). Similar reduction in GPR75 expression was seen in other tissues, including the liver (45%) and heart (80%; Online Figure VI). Elevated vascular ACE has been characterized as a hallmark of the doxycycline-induced 20-HETE–dependent hypertension in Cyp4a12tg mice. In line with previous studies, vascular ACE expression in doxycycline- and doxycycline+control shRNA-treated Cyp4a12tg mice increased by 3.3- and 3.8-fold, respectively (Figure 5D). In contrast, vascular ACE expression was not induced in doxycycline-treated Cyp4a12tg mice that received GPR75-targeted shRNA lentiviral particles (Figure 5D), further substantiating the notion that the GPR75–20-HETE pairing is critical to achieve 20-HETE–mediated induction of ACE.

The hypertensive phenotype of the doxycycline-treated Cyp4a12tg mice is associated with 20-HETE–dependent endothelial dysfunction and enhanced sensitivity to constrictor stimuli. Here, we show that knockdown of GPR75 interferes with the ability of doxycycline to impair relaxations to acetylcholine and increase contractions to phentolamine. The relaxation to acetylcholine was markedly reduced in interlobar arteries from doxycycline-treated (55±3%) when...
compared with arteries from water-treated Cyp4a12tg mice (99±2%). Administration of GPR75-targeted, but not control, shRNA lentiviral particles prevented doxycycline-induced impairment in relaxation to acetylcholine (88%±3%; Figure 6A). Likewise, administration of GPR75-targeted shRNA prevented the doxycycline-induced increases in contractions to phenylephrine. Treatment with doxycycline increased sensitivity to phenylephrine (P<0.05) as evidenced by a reduction in EC\textsubscript{50} (from 0.75±0.11 to 0.40±0.08 µmol/L) and an increase in R\textsubscript{max} (from 4.89±0.51 to 6.17±0.39 mN/mm) when compared with water-treated Cyp4a12tg mice (Figure 6B). However, the EC\textsubscript{50} to phenylephrine in arteries from doxycycline-treated mice that received GPR75-targeted shRNA was unchanged (0.86±0.24 µmol/L) and was not different from the EC\textsubscript{50} in arteries from control mice (Figure 6B).

Remodeling of the renal microvasculature is a striking pathology associated with chronic hypertension. Prolonged exposure of the vasculature to high levels of 20-HETE as in the androgen-treated rats or Cyp4a12tg mice receiving doxycycline leads to hypertrophic remodeling of the renal microvessels in a 20-HETE–dependent manner that is largely independent of blood pressure elevation.\textsuperscript{33,36} In this study, assessment of remodeling in interlobar arteries (80–100 µm) showed that hypertension in Cyp4a12tg mice receiving control shRNA+doxycycline for 35 days was associated with a 3-fold increase in media thickness, media:lumen ratio, and cross-sectional area (Figure 6C through 6F). In contrast, neither hypertension nor microvascular remodeling occurred in arteries from doxycycline-treated Cyp4a12tg mice that received GPR75-targeted shRNA for 35 days was associated with a 3-fold increase in media thickness, media:lumen ratio, and cross-sectional area (Figure 6C through 6F). Taken together, these data strongly support the notion that activation of GPR75 is a necessary step in 20-HETE–mediated hypertension, endothelial dysfunction, vascular smooth muscle contractions, and microvascular remodeling.
20-HETE–GPR75 Pairing in Vascular Smooth Muscle Cells Is Associated With Gαq/11 and GIT1-Mediated Signaling

20-HETE has been shown to stimulate vascular smooth muscle contraction via mechanisms that include activation of protein kinase Cα, mitogen-activated protein kinase, and Src-type tyrosine kinases, all of which can phosphorylate and inhibit the Ca²⁺-activated K⁺ channels (BKca), leading to depolarization, elevation in cytosolic [Ca²⁺], and increased Ca²⁺ entry through the L-type Ca²⁺ channels. As seen in Figure 7, GPR75 is expressed in cultured aortic vascular smooth muscle cells. In these cells, 20-HETE stimulated the dissociation of Gαq from GPR75 (Figure 7A), GPR75–GIT1 association (Figure 7B), GIT1–protein kinase Cα dissociation (Figure 7C), protein kinase Cα-large conductance voltage and calcium-activated potassium subunit β association (Figure 7D), c-Src-large conductance voltage and calcium-activated potassium subunit β association (Figure 7E), and large conductance voltage and calcium-activated potassium subunit β tyrosine phosphorylation (Figure 7F). The phosphorylation of the large conductance voltage and calcium-activated potassium subunit β subunit of the BKca channels leads to the inactivation of the channel and consequently to vasoconstriction.

Discussion

Recent clinical studies and findings in animal models of genetically determined dysfunction identified 20-HETE as a key lipid regulator of vascular, renal, and cardiac functions. Numerous reports documented causal relationships and polymorphic associations between levels of 20-HETE and its biosynthetic enzymes with hypertension, vascular and renal injury, and cardiac hypertrophy. The discovery of a receptor–ligand interaction between 20-HETE and the GPR75 orphan receptor, capable of modulating some of the published effects of 20-HETE in the regulation of vascular function and blood pressure, represents a major breakthrough in this area of research; it provides the molecular basis for the signaling and pathophysiological functions mediated by 20-HETE and is the first identification of a CYOP450–derived eicosanoid receptor.

The presence of a specific cellular receptor/target for 20-HETE has been contemplated since the first demonstration of its occurrence within the renal and cerebral microcirculation and its distinctive roles in the regulation of the myogenic tone. The identification of a specific receptor, responsible for the bioactive properties of 20-HETE, posed significant difficulties as 20-HETE is chemically and metabolically labile and, as a lipid, it can cross membranes, and is rapidly esterified into phospholipids, or effectively binds/sticks to proteins.

Figure 5. G-protein receptor 75 (GPR75) knockdown prevents 20-hydroxyeicosatetraenoic acid (20-HETE)–dependent hypertension and angiotensin-converting enzyme (ACE) induction in Cyp4a12tg mice. A, Systolic blood pressure monitoring in Cyp4a12tg mice receiving water, control shRNA+doxycycline (DOX) and GPR75 shRNA+DOX (n=4, *P<0.05 vs water) for 14 d. B, Systolic blood pressure of Cyp4a12tg mice at day 14 of treatment with water, DOX, control shRNA, control shRNA+DOX, or GPR75 shRNA+DOX. C, Vascular GPR75 expression in preglomerular microvessels from control and GPR75 shRNA-treated Cyp4a12tg mice at day 14. D, Vascular ACE expression in preglomerular arteries from Cyp4a12tg mice receiving water, DOX, control shRNA, control shRNA+DOX, and GPR75 shRNA+DOX for 14 d. Blood pressure was measured by the tail cuff method. Results are mean±SEM, n=4, *P<0.05 vs water or control shRNA+DOX.
overcome some of these difficulties, we used the 20-HETE analog, 20-APheDa, and click chemistry methodology coupled with protein partner identification to expose a multi-protein complex that is associated with 20-HETE binding in EC. The use of 20-APheDa allowed us to take on a broader approach and obtain clues toward identifying receptor candidates. Click-chemistry compounds have been proven to be useful for labeling and used for the identification of multiprotein complexes and interactions.30 In these experiments, we aimed at potentially labeling several proteins in close proximity to 20-APheDa in its bound and docked position. Several in-gel 20-APheDa–protein complexes were observed including a dominant band, which became the focus of our analysis. This band revealed 20-APheDa's effective labeling of several proteins, and it was not until this information was used for protein partner analysis that we were able to identify a candidate receptor GPR75, a member of the rhodopsin Gq receptor family, as the putative GPCR. The ligand–receptor pairing of 20-HETE with GPR75 was further demonstrated by competition binding studies in EC membranes leading to Kd values that are well within the concentration range of 20-HETE biological actions. Moreover, the finding that GPR75-deficient EC do not bind 20-HETE clearly support the hypothesis that GPR75 is the receptor target for 20-HETE in these cells. In line with the fact that GPR75 is a Gαq-coupled receptor are the demonstrations that 20-HETE increases IP-1 accumulation, an effect not present in GPR75-deficient cells, and that 20-HETE rapidly dissociates Gαq from GPR75. All together, these results indicate that 20-HETE binds to EC membranes, pairs with GPR75, and functionally activates it.

In 1999, Tarttelin et al39 identified GPR75 as a novel human GPCR that maps to chromosome 2p16 and encodes a 540 amino acid protein. Initial findings showed GPR75 to be predominantly expressed in cells surrounding retinal arterioles and in other areas of the brain.39,40 Numerous databases indicated a broad expression profile for Gpr5 in the majority of human tissues, and our qPCR assessment in tissues from C57BL/6 corroborated this expression profile. Ignatov et al35 reported that the chemokine CCL5 increased IP3 and intracellular Ca2+ in CHO (Chinese hamster ovary) or HEK (human embryonic kidney) cells overexpressing the mouse GPR75 via a Gq-protein–coupled phospholipase C (PLC)-mediated signal transduction. No direct binding studies were documented in that study. A recent study by Liu et al41 showed that CCL5 stimulates insulin secretion in isolated islets via PLC-activated Ca2+ influx in a GPR75-dependent manner; however, they also did not document direct binding or interaction between GPR75 and CCL5. Importantly, the pairing of CCL5 and GPR75 could not be repeated in a recent β-arrestin assay and the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification still classifies GPR75 as an orphan receptor.42,43 Notably, in our hands, CCL5 failed to dissociate Gαq from GPR75 in EC. Moreover, CCL5 lacked the ability to transactivate EGFR.

Figure 6. G-protein receptor 75 (GPR75) knockdown prevent 20-hydroxyeicosatetraenoic acid (20-HETE)–dependent hypertension, vascular dysfunction, and vascular remodeling (A–F). A, Cumulative concentration–response curve to acetylcholine in renal interlobar arteries from Cyp4a12tg mice treated with water, control shRNA, doxycycline (DOX), control shRNA+DOX, and GPR75 shRNA+DOX for 14 d (mean±SEM, n=4, *P<0.05 vs water, #P<0.05 vs control shRNA+DOX). B, Cumulative concentration–response curve to phenylephrine in renal interlobar arteries from water, control, and GPR75 shRNA-treated mice receiving DOX after 12 d. EC50 and Rmax are given in the upper inset (n=6, *P<0.05 vs water). C, Systolic blood pressure measurements (tail cuff) in control and GPR75 shRNA-treated Cyp4a12tg mice receiving DOX for 35 d (n=3, *P<0.05 vs water, #P<0.05 vs control shRNA+DOX). Vascular remodeling measured by pressure myography as (D) media thickness, (E) media:lumen ratio, and (F) cross-sectional area in renal interlobar arteries from mice treated with control and GPR75 shRNA after receiving DOX for 35 d as described previously29 (n=4, *P<0.05 vs water, #P<0.05 vs control shRNA+DOX).
and induce ACE mRNA. 2 bioactions of 20-HETE that are initiated with the activation of GPR75. Nevertheless, we cannot exclude the possibility of interactions between CCL5 and 20-HETE at the level of GPR75 whereby CCL5 facilitates, amplifies, or hinders the binding of 20-HETE.

We proposed GPR75 as a 20-HETE receptor based on 2 key original findings. First, when applied to EC, 20-HETE binds and activates the receptor at a concentration that elicits a specific EGFR-mitogen-activated protein kinase signaling pathway to induce ACE transcription, uncouple endothelial NO synthase, and stimulate cytokine production. This activation involves dissociation of Gαq/11 from GPR75 and increased GIT1–GPR75 association followed by c-Src-mediated 20-HETE–dependent EGFR phosphorylation, the first step of 20-HETE signaling in EC. Moreover, both GPR75 and GIT1 are required not only for 20-HETE–mediated transactivation of EGFR but also for the downstream effect of 20-HETE to induce ACE in EC (Figure 8). The dissociation of Gαq/11 after the addition of 20-HETE, most likely, initiated a PLC-IP-3–mediated increases in [Ca2+] as a common signaling pathway for Gq-coupled receptor. Hence, the demonstration that 20-HETE increases IP-1 levels in a GPR75-dependent manner further indicates a functional GPR75–20-HETE pairing. In smooth muscle cells, inhibition of the BKca channels underlies 20-HETE–mediated vasoconstriction. Here, we link GPR75–20-HETE pairing to stimulation of Gαq/11 dissociation and GIT1-mediated protein kinase Cα– and cSrc-stimulated tyrosine phosphorylation of large conductance voltage and calcium-activated potassium subunit β, which inactivates the channel and alters BKca subunit trafficking leading to cell depolarization, elevation in cytosolic [Ca2+], and stimulation of the contractile apparatus. The role of HIC-5 in the vascular endothelium is unclear. HIC-5 has been shown to interact with GIT1 and coactivates the androgen receptor. The interaction between HIC-5 and the androgen receptor may account for the strong relationship between androgens and 20-HETE.

The second key finding in this study is that the expression of GPR75 is required for the 20-HETE prohypertensive activities. The conditional Cyp4a12tg mice display doxycycline-mediated hypertension along with vascular dysfunction and remodeling in a 20-HETE–dependent manner. 20-HETE–mediated vascular dysfunction and remodeling has been shown to be largely independent of blood pressure elevation. We hypothesized that if GPR75 is the 20-HETE receptor; its disruption should prevent hypertension in the doxycycline-treated Cyp4a12tg mice. Indeed, knockdown of GPR75 in doxycycline-treated Cyp4a12tg mice prevented the 20-HETE–dependent hypertension along with marked reduction in endothelial dysfunction, smooth muscle contractility, and vascular remodeling. These results clearly place GPR75 as a novel target in the control of blood pressure and vascular function. Until recently, there were no observed associations...
between GPR75 and hypertension. There is, however, a report that identified GPR75 among other potential candidate genes in a locus on chromosome 2p, which shows significant linkage to antihypertensive responses in the British Genetics of Hypertension Study.51

As indicated above, GPR75 has been shown to express in a wide variety of tissues including the brain, heart, and kidney. All of these tissues participate in blood pressure control and have been shown to have the capacity to produce 20-HETE. Accordingly, one may expect the presence of 20-HETE–GPR75 pairing in these tissues that may also contribute to hypertension. To this end, we found that GPR75 is also expressed along the nephron, primarily on the luminal side of the proximal tubules (unpublished data) where 20-HETE is presumably activating the NHE3 via angiotensin–dependent mechanisms.52 Interestingly, salt-sensitive hypertension in mice expressing the human CYP4A11–20-HETE synthase was attributed to enhanced Na retention resulting from 20-HETE– and Ang II–dependent NCC upregulation.53 Salt-sensitive hypertension was also shown in rats with depressed renal medullary 20-HETE synthesis, which conditions Na retention in the ascending limb of the loop of Henle.54–57 Inasmuch as 20-HETE contributes to prohypertensive mechanisms via vascular and tubular actions that foster vasoconstriction and conservation of sodium, and to antihypertensive mechanisms via actions that facilitate Na excretion, it is expected that the blood pressure effect of interventions that interfere with 20-HETE actions is conditioned by both mechanisms. The discovery of 20-HETE–GPR75 pairing provides a unique opportunity to confront the seemingly opposing actions of 20-HETE on blood pressure and explore the contribution of GPR75–20–HETE interactions in other tissues, including the central nervous system, to hypertension.

In summary, we provide strong evidence for a 20-HETE–GPR75 pairing in the vascular endothelium. We also present substantial data demonstrating that GPR75 is a critical

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**Figure 8.** Proposed 20-hydroxyeicosatetraenoic acid (HETE)–G-protein receptor 75 (GPR75)–mediated signaling in endothelial cells. 20-HETE–GPR75 pairing stimulates the dissociation of Gαq/11 and the association of G-protein–coupled receptor-kinase interacting protein-1 (GIT1) to the receptor. The latter facilitates c-Src–mediated endothelial growth factor receptor (EGFR) transactivation. The 20-HETE–GPR75–mediated activation of EGFR results in the stimulation of downstream cascades that regulate vascular angiotensin-converting enzyme (ACE) expression and decreases in nitric oxide (NO) bioavailability. DAG indicates diacylglycerol; eNOS, endothelial nitric oxide synthase; HIC-5, hydrogen peroxide–inducible clone 5; HSP, heat shock protein; IKK, inhibitor of nuclear factor κ-B kinase subunit β; IP, inositol phosphate; NF, nuclear factor; PKC, protein kinase C; and PLC, phospholipase C.
component of 20-HETE’s signaling and bioactions in vitro and in vivo, regulating vascular tone, and function. This finding opens the door for discovery, and there is much to be explored including the role of this pairing in other sites and organs such as the nervous, endocrine, and respiratory systems. It has the potential to be a game changer in the field of CYP eicosanoids by providing novel targets for the development of new therapies for myriad diseases/pathologies associated with 20-HETE (e.g., stroke and myocardial infarction), whereas genetic studies of receptor variants could lead to a better understanding of some discrepancies between 20-HETE levels and reported pro- or antihypertensive properties.

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20-HETE Signals Through G-Protein–Coupled Receptor GPR75 (Gq) to Affect Vascular Function and Trigger Hypertension


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SUPPLEMENTAL MATERIAL

20- HETE signals through G protein-coupled receptor GPR75 (Gq) to affect vascular function and trigger hypertension

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Material and Methods

**Synthesis of Photoactivated Cross-Linker 20-azido-N-((4-(3-(4-benzoylphenyl)propanamido)-phenyl)sulfonyl)-19(S)-hydroxyeicosa-5(Z),14(Z)-dienamide (20-APheDa).**

Nuclear magnetic resonance (NMR) spectra were recorded on Varian 300, 400 or 500 spectrometers at operating frequencies of 300/400/500 MHz ($^1$H) or 75/100/125 MHz ($^{13}$C) in CDCl$_3$ with TMS as internal standard, unless otherwise stated. $^1$H NMR data are reported as follows: chemical shift, $^1$ multiplicity (s = singlet, br s = broad singlet, d = doublet, t = triplet, q = quartet, app q = apparent quartet, qn = quintet, app qn = apparent quintet, m = multiplet) and coupling constant (Hz). High resolution mass spectra (HRMS) were obtained at UT-Arlington using a Shimadzu IT-TOF mass spectrometer or at the Medical College of Wisconsin by Prof. Michael Thomas. Infrared (IR) spectra were obtained using a Perkin Elmer Spectrum 1000 Fourier transform spectrometer. Melting points were measured using an OptiMelt from Stanford Research Systems and are uncorrected. Analytical thin layer chromatography (TLC) used EMD Chemicals TLC silica gel 60 F254 plates (0.040-0.063 mm) with visualization by UV light and/or KMnO$_4$ or phosphomolybdic acid (PMA) solution followed by heating. All oxygen and/or moisture sensitive reactions were performed under an argon atmosphere using oven-dried glassware and anhydrous solvents. Extracts were dried over anhydrous Na$_2$SO$_4$ and filtered prior to removal of all volatiles under reduced pressure. Chromatographic purifications utilized preparative TLC or flash chromatography using pre-packed SiO$_2$ columns on a CombiFlash® R,200 chromatograph (Teledyne Isco). Unless otherwise noted, yields refer to isolated and purified material with spectral data consistent with assigned structures or, if known, were in agreement with published data. Reagents were purchased at the highest commercial quality available and used without further purification, unless otherwise noted. Anhydrous solvents were dried using a Glass Contours Solvent System by passage through columns of activated packing material under argon immediately prior to use.

![Chemical Structure](image)

Solid 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI-HCl) (255 mg, 1.33 mmol) was added in portions to a stirring, rt solution 3-(4-benzoylphenyl)propanoic acid (200 mg, 0.786 mmol), 2 commercial p-sulfanilamide (135 mg, 0.786 mmol), and hydroxybenzotriazole (HOBT) (116 mg, 0.865 mmol) in anhydrous DMF (5 mL). After 18 h, the reaction mixture was diluted with water (20 mL). The resultant precipitate was collected by filtration, washed with water (30 mL) and dried in vacuo at 30 °C to give 3-(4-benzoylphenyl)-N-(4-sulfamoylphenyl)propanamide (224 mg, 70%) as a white powder. TLC: 80% EtOAc/hexanes, $R_f = 0.5$.

$^1$H NMR (400 MHz, DMSO) $\delta$ 10.29 (s, 1H), 7.82–7.64 (m, 9H), 7.57 (d, $J = 8.0$, 2H), 7.45 (d, $J = 8.0$, 2H), 7.24 (s, 2H), 3.03 (t, $J = 7.6$, 2H), 2.75 (d, $J = 7.6$, 2H).

![Chemical Structure](image)

Imidazole (59 mg, 0.86 mmol), TBDMS-Cl (52 mg, 0.34 mmol), and DMAP (5 mg) were added to a stirring, 0 °C solution of methyl 20-azido-19(S)-hydroxyeicosa-5(Z),14(Z)-dienoate$^a$ (110 mg, 0.28 mmol) in dry CH$_2$Cl$_2$ (15 mL). After stirring at room temperature for 48 h, the reaction mixture was quenched with water (5 mL) and extracted with CH$_2$Cl$_2$ (3 × 6 mL). The combined
organic extracts were washed with brine (10 mL), dried over Na₂SO₄, and concentrated under reduced pressure. The residue was purified by SiO₂ column chromatography using hexanes/EtOAc (94:6) to give methyl 20-azido-19(S)-(tert-butyldimethylsilyloxy)eicosa-5(Z),14(Z)-dienoate (134 mg, 94%) as a colorless oil. TLC: 20% EtOAc/hexane, Rₛ ≈ 0.6.¹H NMR (CDCl₃, 400 MHz) δ 5.45–5.24 (m, 4H), 3.76–3.65 (m, 1H), 3.65 (s, 3H), 3.23 (dd, J = 12.4, 4.1 Hz, 1H), 3.12 (dd, J = 12.5, 5.9 Hz, 1H), 2.30 (t, J = 7.5 Hz, 2H), 2.11–1.92 (m, 8H), 1.74–1.63 (m, 2H), 1.58–1.43 (m, 2H), 1.43–1.22 (m, 12H), 0.89 (s, 9H), 0.09 (s, 3H), 0.07 (s, 3H); ¹³C NMR (CDCl₃, 101 MHz) δ 174.07, 131.09, 130.50, 129.05, 128.31, 71.69, 56.62, 51.42, 34.65, 33.42, 29.70, 29.67, 29.43, 29.26, 27.25, 27.19, 27.15, 26.51, 25.77, 25.21, 24.86, 17.99, -4.64, -4.67.

A solution of LiOH (0.8 mL of 1 M aq. soln, 0.78 mmol) and methyl 20-azido-19(S)-(tert-butyldimethylsilyloxy)eicosa-5(Z),14(Z)-dienoate (130 mg, 0.26 mmol) in THF (4 mL) and deionized H₂O (1 mL) was stirred at rt for 16 h, then the organic solvent was evaporated under reduced pressure. The resultant aqueous solution was acidified to pH 4.5 with 1N HCl (2 mL) at 0 °C and extracted with EtOAc (3 × 10 mL). The organic extracts were washed with brine (10 mL), dried over Na₂SO₄, and concentrated under reduced pressure. The residue was purified by SiO₂ column chromatography eluting with a gradient of 0-5% IPA/hexanes to afford 20-azido-19(S)-(tert-butyldimethylsilyloxy)eicosa-5(Z),14(Z)-dienoic acid (114 mg, 90%) as a colorless oil. TLC: 60% EtOAc/hexane, Rₛ ≈ 0.5.¹H NMR (CDCl₃, 400 MHz) δ 5.47–5.25 (m, 4H), 3.79–3.10 (m, 1H), 3.24 (dd, J = 12.4, 4.1 Hz, 1H), 3.13 (dd, J = 12.5, 5.9 Hz, 1H), 2.35 (t, J = 7.5 Hz, 2H), 2.14–1.93 (m, 8H), 1.70 (p, J = 7.5 Hz, 2H), 1.59–1.44 (m, 2H), 1.44–1.23 (m, 12H), 0.90 (s, 9H), 0.10 (s, 3H), 0.08 (s, 3H); ¹³C NMR (CDCl₃, 101 MHz) δ 180.18, 131.30, 130.52, 129.06, 128.12, 71.71, 56.62, 34.65, 33.43, 29.71, 29.67, 29.43, 29.27, 29.26, 27.26, 27.22, 27.16, 26.42, 25.79, 25.22, 24.58, 18.01, -4.63, -4.66.

A mixture of 3-(4-benzoylphenyl)-N-(4-sulfamoylphenyl)propanamide (26 mg, 0.06 mmol), EDCI·HCl (7 mg, 0.045 mmol), and DMAP (6 mg, 0.045 mmol) was subjected to high vacuum for 30 min at rt. The mixture was then dissolved in dry DMF (1 mL) and a solution of 20-azido-19(S)-(tert-butyldimethylsilyloxy)eicosa-5(Z),14(Z)-dienoic acid (20 mg, 0.04 mmol) in anhydrous DMF (1 mL) was added. After 12 h, the mixture was charged with water (10 mL) and
extracted with EtOAc (3 × 30 mL). The organic layer was washed with water (5 × 2 mL), brine (10 mL), dried over Na₂SO₄, and concentrated under reduced pressure. The residue was purified by SiO₂ column chromatography eluting with a gradient of 50-70% EtOAc/hexanes to afford 20-azido-N-((4-(3-(4-benzoylphenyl)propanamido)phenyl)sulfonyl)-19(S)-(tert-butyldimethylsilyloxy)eicosa-5(Z),14(Z)-dienamide (25 mg, 70%) as a waxy solid. TLC: 60% EtOAc/hexanes, Rₜ ≈ 0.45.

¹H NMR (500 MHz, CDCl₃) δ 8.03–7.99 (m, 2H), 7.96 (s, 1H), 7.82–7.74 (m, 4H), 7.71–7.64 (m, 2H), 7.52–7.47 (m, 2H), 7.44 (s, 1H), 7.39–7.33 (m, 2H), 7.28 (t, J = 7.5 Hz, 2H), 2.23 (t, J = 7.5 Hz, 2H), 2.05–1.95 (m, 8H), 1.65–1.63 (m, 2H), 1.55–1.20 (m, 2H), 1.36–1.25 (m, 12H), 0.91 (s, 9H), 0.11 (s, 3H), 0.09 (s, 3H).

Cell Culture. Human microvascular or umbilical vein endothelial cells (Lonza Inc., Allendale NJ) were used in this study. Both responded equally to 20-HETE with respect to GPR75 activation and are referred as EC throughout the manuscript. Cells were grown in Medium 131 containing growth supplement (Invitrogen, Waltham, MA). Passages 2-3 were used for all experiments. For the aortic vascular smooth muscle cell experiments, A7r5 cells were used (ATCC, Manassas,
VA). All cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂:95% O₂. For most experiments, cells were grown in 6-well-plates to 80-90% confluence and placed in serum-free (Hank's Buffered Salt Solution, HBSS) media for 12 h prior to addition of 20-hydroxy-5,8,10,14-eicosatetraenoic acid (20-HETE, 10 nmol/L) with and without the 20-HETE antagonist N-(20-hydroxyeicosa-6(Z), 15(Z)-dienoyl) glycine (20-HEDGE).

**Click-Chemistry.** Cell membranes were prepared using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, Grand Island, NY). Cell extracts (20 µg, 10µl) were incubated with 10 nmol/L (5 µl) of the photoactive cross linker 20-azido-N-((4-(3-(4-benzoylphenyl)propanamido)phenyl)sulfonyl)-19(S)-hydroxyeicosa-5(Z),14(Z)-dienamide (20-APheDa) in 6 x 50 mm borosilicate glass culture tubes. Samples were exposed to 365 nm long wave UV light for 15 min in a Stratalinker 2400 (Stratagene, La Jolla, CA). After photocrosslinking, 5 µl of 50 µmol/L of dibenzocyclooctyne (DBCO) 800CW Infrared Dye (LI-COR, Lincoln, NE) was added to each sample and incubated for 1 h. The DBCO IRDye preferentially reacts with the azide functional group forming a covalent bond. Loading dye (x4; 5 µl) containing glycerol (40%), Tris/HCl pH 6.8 (240 mM), SDS (8%), bromophenol blue (0.04%) and beta-mercaptoethanol (5%) was added to each sample (final volume, 25 µl) and samples were run on a 4-20% Mini-PROTEAN TGX precast gel (Bio-Rad, Hercules, CA). Each sample contained 5 µl loading dye. Samples did not undergo boiling or changes in temperature in order to prevent changes in 20-APheDa binding signal. Excess heat has been characterized as potentially disruptive in several click-compound assays. In-gel sample-complex IRDye signal was detected using the LI-COR Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) and Odyssey Application Software Version 3.0.21. Upon visualization, gels were marked and respective bands were extracted, placed in tubes containing 100 µl de-ionized water and sent for protein sequencing to Applied Biomics (Hayward, CA). Protein identification of the dominant band location was based on peptide fingerprint mass mapping (using MS data) and peptide fragmentation mapping (using MS/MS data). The MASCOT search engine was used to identify proteins from primary sequence databases. Protein partner analysis of the top protein hits were conducted using STRING and NCBI protein partner databases. For competition experiments, 10 µg of HMVEC cell membranes were incubated with 0.1 nmol/L of 20-APheDa in the presence and absence of 100 µmol/L 20-HETE or 100 μmol/L 12-(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE) for 15 min prior to UV photocrosslinking and the addition of DBC IRDye. In-gel visualization was assessed using the LI-COR Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) and Odyssey Application Software Version 3.0.21.

**Synthesis of [5,6-;8,9-;11,12-;14,15-3H]20-hydroxyeicosatetraenoic acid [3H8] 20-HETE.** Radiolabeled [3H8] 20-HETE was synthesized as follows. A mixture of 50 µl [5,6,8,9,11,12,14,15-3H] arachidonic acid (AA) ([50 µCi labeled [3H8] AA at ~50% efficiency = 50 x 10⁶ cpm) (Moravek Biochemicals, Brea, CA) with 7.5 nmols of unlabeled AA (Cayman Chemicals, Ann Arbor, MI) for a final specific activity of 6 x 10³ cpm/pmol was prepared. The AA mixture was dried under N₂ in the incubation vial. The enzyme mixture including reconstituted 20-HETE synthase composed of purified recombinant CYP4A11 0.25 μmol/L, CYP450 Oxido-Reductase (P450-OR) 2.5 μmol/L and Cytochrome b₅ (b₅) 2.5 μmol/L (mixed in a 1:10:1 molar ratio) in the presence of 50 ug/ml dilauroyl-phosphatidylcholine (DLPC) (Sigma, St Louis, MO) was mixed and incubated for 10 minutes. After the incubation, 170 µl of buffered NADPH generating mix containing: 50 mmol/L Tris-Cl pH 7.4 containing 10 mmol/L MgCl₂ (Sigma, St Louis, MO), 2 mg/ml Sodium Isocitrate (Sigma, St Louis, MO), 0.2 IU/ml Isocitrate dehydrogenase (Sigma, St Louis, MO), and 1 mmol/L 1,4 dithio-D-threitol (DTT) (Sigma, St Louis, MO) was added to the dried AA mixture followed by the addition of the pre-incubated enzyme mixture. The mixture was kept for 5 minutes at 37°C with gentle mixing. The reaction was started by adding 10 µl of 20 mmol/L NADPH (EMD Millipore, Billerica, MA) and stopped...
after 10 min at 37°C by the addition of 200 μl of CH₃CN 0.5% CH₃COH (AcOH) (Sigma, St Louis, MO). The mixture was vortexed and spun at full speed in a mini-centrifuge. The supernatant was collected and dried under N₂. A pasteur pipette SiO₂ column (~2.5 x 0.5 cm) was made and equilibrated with Hexane/0.5% AcOH. The dried sample was dissolved in 2 ml of Hexane/0.5% AcOH (Sigma, St Louis, MO) and loaded onto the column. The column was washed with 10 ml Hexane/0.5% AcOH. The sample was eluted with 5-10 ml of 50% Ethyl Ether/50% Hexane containing 0.5% AcOH (Sigma, St Louis, MO). The eluted sample was then dried under N₂ and saved in -80°C for HPLC purification. The total CPM in the purified [³H] 20-HETE sample was assessed using a Hidex 300 SL liquid scintillation counter (Hidex, Turku, Finland).

[³H] 20-HETE Binding Assays. Cells were harvested by trypsinization followed by centrifugation and the cell pellet was resuspended in Hanks’ balanced salt solution containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). These experiments followed a similar protocol by Chen et al. Briefly, cells were then sonicated (20-s bursts) on ice five times using a Q500 Sonicator (QSonica Newton, CT). The homogenate was centrifuged at 1000g for 10 min at 4°C to remove unbroken cells. The supernatant was centrifuged at 150,000g for 45 min at 4°C. The pellet represents the membrane fraction and was resuspended in HBSS containing 50 mmol/L HEPES, pH 7.4. For dissociation studies, 50 μg of HMVEC membrane fraction was incubated with [³H] 20-HETE (8000 CPM equivalent to 6.67 nmol/L) in the presence and absence of increasing concentrations of unlabeled 20-HETE or 12(S)-HETE (0.01-100 nmol/L) for 15 min. Incubations were terminated by filtration through GF/B glass filter paper (Whatman, Clifton, NJ), followed by five washes with 5 ml of ice-cold binding buffer consisting of 20 mmol/L HEPES, 10 mmol/L CaCl₂, and 10 mmol/L MgCl₂, at pH 7.4. Radioactivity ([³H] 20-HETE bound to membrane proteins attached to filter paper) was assessed using a Hidex 300 SL liquid scintillation counter (Hidex, Turku, Finland).

Transfection with siRNA. Cells were grown on 6-well plates to 70% confluence. Cells were washed with siRNA transfection medium (Santa Cruz, Dallas, TX) and incubated with a mixture of GPR75 (sc-94341), GIT1 (sc-35477) or Control (sc-37007) siRNAs (Santa Cruz, Dallas, TX) and siRNA transfection reagent (Santa Cruz, Dallas, TX) for 6 h following manufacturer’s instructions. The transfection solutions were replaced with fresh growth medium for an additional 12 h until 80-90% confluence. Cells were then placed in HBSS for an additional 12 h and treated with 20-HETE (10 nmol/L) for 5 min for immunoprecipitation studies or 2h for ACE mRNA expression analysis by real-time PCR. In addition, membranes from control- and GPR75-specific siRNA-transfected cells were prepared for binding assay as described above.

IP-One assay. IP-1, a downstream metabolite of IP3, accumulates in cells following Gq receptor activation and has been used as a functional assay for Gq-GPCR. The assay used the IP-One HTRF assay kit (Cisbio, Beford, MA) and was performed according to the manufacturer’s instructions. Briefly, cells were seeded into 96-well tissue culture treated white microplates and grown in regular growth media at 37°C in a 5% CO₂:95% O₂ atmosphere for 24 h before the assay. Media was then removed, 20-HETE prepared in stimulation buffer containing LiCl (50 mmol/L) to prevent IP-1 degradation was added and cells were incubated for 5 min. The IP1-d2 conjugate in lysis buffer was added to wells followed by addition of anti-IP1-Cryptate antibody. Plates were incubated for 1 hour at room temperature and then read on Synergy HT plate reader (BioTek, Winooski, VT) using excitation at 320 nm and emissions at both 620nm and 665nm. The ratio of 665nm/620nm emissions was calculated. A standard curve of the ratio 665/620 versus log 1P-1 concentration was plotted using a non-linear least squares fit (sigmoidal dose response variable slope, 4PL) and unknowns were read from standard curve as nM concentration of IP1.
**Immunoprecipitation.** Cells were cultured on 6-well plates to 80-90% confluence and starved in serum-free media for 12 h. Cells were treated with 20-HETE (10 nmol/L) or its vehicle (PBS) for 5 min. In some experiment, cells were incubated with CCL5 (1 nmol/L) for 5 min. Cells were lysed with 1X RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma, St Louis, MO) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Protein concentrations were determined using the Bradford protein assay (Eppendorf BioPhotometer). Immunoprecipitation was conducted using the Dynabeads Protein G Immunoprecipitation Kit (Life Technologies, Grand Island, NY). Dynabeads were incubated with primary antibodies against human EGFR antibody (AHR5062, Invitrogen, Camarillo, CA, 175 kDa), GPR75 (sc-164538, Santa Cruz, Biotechnology, Dallas, TX, 55 kDa), and GIT1 (sc-9657, Santa Cruz, Biotechnology, Dallas, TX, 95 kDa), for one hour prior to washing and incubation with 5 µg of HMVEC cell lysate overnight. Samples were then washed and eluted per manufacturer's protocol and loaded onto a 4-20% Mini-PROTEAN TGX precast gel (Bio-Rad, Hercules, CA) and transferred using the Trans-Blot® Turbo™ transfer system to a PVDF membrane. Immunoblotting for respective associated proteins was conducted using phosphorylated tyrosine antibody (SC-7020, Santa Cruz, Biotechnology, Dallas, TX), EGFR antibody (AHR5062, Invitrogen, Camarillo, CA, 175 kDa), GPR75 (sc-164538, Santa Cruz, Biotechnology, Dallas, TX, 55 kDa), GIT1 (sc-9657, Santa Cruz, Biotechnology, Dallas, TX, 95 kDa), HIC-5 (sc-17616, Santa Cruz, Biotechnology, Dallas, TX, 48 kDa), G alpha q/11 (sc-392, Santa Cruz, Biotechnology, Dallas, TX, 40-41 kDa), c-SRC (sc-19, Santa Cruz, Biotechnology, Dallas, TX, 60 kDa). For vascular smooth muscle cell experiments, the PKCα (C-20) sc-208 and MaxiKβ (I-191) sc-33608 (Santa Cruz, Biotechnology, Dallas, TX, 80 and 26-37 kDa, respectively) were used. Membrane fluorescence-based immunodetection was conducted using the appropriate LI-COR secondary IRDye antibody and LI-COR Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Respective band density was quantified using the Odyssey Application Software Version 3.0.21. The GPR75 antibody was a polyclonal antibody against the c-terminal; it recognized a single 54-55 KDa protein band as shown in supplementary Fig. 2.

**Western Blot Analysis.** Samples were lysed with 1X RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma, St. Louis, MO) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Protein concentrations were determined using the Bradford protein assay (Eppendorf BioPhotometer). Samples (20 µg) were run on a 4-20% Mini-PROTEAN TGX precast gel (Bio-Rad, Hercules, CA) and transferred using the Trans-Blot® Turbo™ transfer system to a PVDF membrane. Primary antibodies included: ACE (sc-12184, Santa Cruz, Biotechnology, Dallas, TX, 195 kDa) and β-Actin monoclonal IgG (Sigma, St Louis, MO, 42 kDa). Membrane fluorescence-based immunodetection was conducted using the appropriate LI-COR secondary IRDye antibody and LI-COR Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Respective band density was quantified using the Odyssey Application Software Version 3.0.21.

**SYBR Green qPCR and Analysis.** cDNAs synthesized from a panel of various C57Bl/6 mouse tissues previously reported were used for the tissue distribution assessment of mouse Gpr75. A specific primer set of oligonucleotides was designed for the single coding exon of Gpr75. The forward primer (5'-AAGCAGCCTAATTGTACAGCCT-3') and the reverse primer (5'-TCCCTTCCCATCATAAGACT-3') were each diluted to a concentration of 10 µM, and 0.5 µl was used for each 10 µl qPCR reaction along with 5 µl of Power SYBR Green PCR Master Mix (Life Technologies (Carlsbad, CA). Samples were run in triplicate on an Applied Biosystems ViiA7 Real-Time PCR System (Life Technologies) with PCR conditions used as follows: 50°C for 2 minutes; 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, then 60°C for 1 minute; a dissociation stage of 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15
seconds. Data was analyzed using the \( 2^{-\Delta \Delta C_T} \) method \(^8\) with the mouse tissue expression values normalized to glyceraldehyde 3-phosphate dehydrogenase (\( Gapdh \)). Outliers as determined by the Grubbs Outlier test \(^9\) were removed. The GraphPad Prism 6.0C program (GraphPad Software, San Diego, CA) was used to generate the tissue expression graph. For determination of ACE expression in EC, cells were cultured on 6-well plates to 80-90% confluence and starved in serum-free HBSS media for 24 h and treated with 20-HETE (10 nmol/L) for 2 h in HBSS. Cells were washed with 1X PBS followed by lysis buffer (Denville Scientific, Metuchen, NJ). Total RNA was isolated using the SpinSmart RNA Purification kit (Denville Scientific, Metuchen, NJ) and quantified using the Synergy HT Take3 Microplate Reader (BioTek, Winooski, VT). RT reaction of total RNA (500 ng) was performed using the qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). Sequences for PCR primers used are as follows: angiotensin converting enzyme (ACE) sense: 5'- TCC CAT CCT TTC TCC CAT TTC -3'; ACE antisense: 5'- GCT CTC CCA ACA CCA CAT TA -3'; 18S rRNA sense: 5'- GAT GGG CGG CGG AAA ATA G -3'; 18S rRNA antisense: 5'- GCG TGG ATT CTG CAT AAT GGT - 3'. Quantitative Real-Time PCR was performed using the PerfeCTa SYBR Green FastMix Low ROX Kit (Quanta Biosciences, Gaithersburg, MD) and the Mx3000p Real-Time PCR System (Stratagene, Santa Clara, CA) as previously described.\(^{10}\)

**TaqMan qPCR.** The Mm00558537_s1 Gpr75 TaqMan primer/probe and the Mm99999915_g1 \( Gapdh \) primer/probe were purchased from Life Technologies. For all qPCR reactions 0.5 \( \mu l \) of the 20X primer/probes and 5 \( \mu l \) of 2X Maxima Probe/ROX qPCR Master Mix (Thermo Scientific, Pittsburgh, PA) were used in a 10 \( \mu l \) final reaction volume. 1 \( \mu l \) of 1 ng/\( \mu l \) diluted tissue cDNAs were used for each reaction. The PCR conditions were as follows: 50°C for 2 minutes; 95°C for 10 minutes, followed by 40 repeat cycles of 95°C for 15 seconds, and 60°C for 1 minute. Samples were run in triplicate.

**Animal Studies.** All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) and by the Institutional Biosafety Committee. The generation and phenotypic characterization of the Cyp4a12 transgenic mice in which the expression of the Cyp4a12-20-HETE synthase is under the control of an androgen-independent, tetracycline (doxycycline, DOX)-sensitive promoter have been previously described \(^{11}\). Male Cyp4a12tg mice (8-14-week-old) were used in all experiments. DOX (1 mg/ml drinking water) was administered to promote the activation of the sole murine 20-HETE synthase, Cyp4a12. Blood pressure was monitored by radiotelemetry and tail cuff before and after administration of DOX for 14-35 days as described below. At the end of all experiments, mice were anesthetized with ketamine (70 mg/kg) and xylazine (70 mg/kg) and laparotomy was performed. Renal preglomerular arteries (the whole vascular tree) or segments of the interlobar arteries (~80-100 \( \mu m \)) were microdissected and collected for western blot analysis and functional studies.

**GPR75 SMARTvector Lentiviral shRNA Studies.** All lentiviral constructs were made by Dharmaco (GE). The SMARTvector Lentiviral shRNA used included non-targeting hCMV-TurboGFP shRNA (VSC7420) and lentiviral hCMV-TurboGFP shRNA particles targeted at GPR75 (V3SM7603 Seq: GGG TTG GCT TCA TGG TTT C). Cyp4a12tg mice were anesthetized under isoflurane and administered lentiviral constructs via a single retro-orbital injection to maximize systemic distribution.\(^{12}\) Each mouse was injected with 100 \( \mu l \) of either 4.33x10\(^9\) TU/mL of non-targeting or 7.29x10\(^9\) TU/mL of GPR75-targeted shRNA particles. The administration of DOX (1 mg/ml) was initiated 48 h after the lentiviral injection. For western blotting, renal preglomerular microvessels were collected and lysed with 1X RIPA buffer (Sigma, St Louis, MO) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Protein concentrations were determined using the Bradford protein assay (Eppendorf BioPhotometer). Protein samples (20 \( \mu g \)) were loaded onto a 4-20% Mini-
PROTEAN TGX precast gel (Bio-Rad, Hercules, CA) with respective loaded EZ-Run Prestained Rec Protein Ladder (Fisher BioReagents, Waltham, MA) markers. SDS-polyacrylamide gels were transferred to Tran-Blot Turbo Mini PVDF membranes (Bio-Rad, Hercules, CA) followed by blocking buffer (Li-Cor, Lincoln, NE) and subsequent incubation with primary and secondary antibodies.

Immunofluorescence Imaging. Liver tissue from mice receiving non-targeting lentiviral hCMV-TurboGFP shRNA particles was harvested to assess GFP transduction. Kidney tissue from C57BL/6 was harvested to examine vascular endothelial GPR75 expression. Tissue samples were fixed in 4% paraformaldehyde at 4°C. Tissues were embedded in cassettes using Tissue-Tek Optimum Cutting Temperature compound. Tissue sections were cut at 3-4 µm thickness. Goat anti GPR75 (sc-164538) and rabbit anti-CD31 antibodies were used as primary antibodies and were from Santa Cruz, Biotechnology, Dallas, TX. Alexa Fluor® 555-conjugated donkey anti-goat and Alexa Fluor® 488-conjugated goat anti-rabbit IgG were used as secondary antibodies and were from Abcam (Cambridge, MA). Immunofluorescence of GFP, GPR75 and CD31 was visualized using a Zeiss Axio Imager M1 fluorescent microscope. Images were captured using AxioVision multichannel image processing software.

Blood Pressure Measurements. Systolic blood pressure measurements in Cyp4a12tg mice were taken using the CODA tail-cuff system (Kent Scientific, Torrington, CT), which utilizes volume pressure recording sensor technology. Mice were acclimated to the machine for one week prior to day 0 and blood pressure was monitored throughout the length of the experiment. Values within ± 10% of their mean blood pressure measurements were obtained. In one set of experiments (n=1/group), blood pressure measurements were conducted in the conscious state by radio-telemetry without distress to the animal after post-surgical recovery. The radio-telemetric system from Data Science International (DSI, St Paul, MN) was used for this procedure. Systolic blood pressure was continuously acquired by implantation of telemetric probe (model TA11PA-C10) into the aorta via the left carotid artery. After one week of recovery from the surgical procedure, blood pressure readings were recorded every ten minutes using Dataquest ART software purchased from Data Science International (DSI). Baseline blood pressure was collected for four days prior to and during the administration of DOX (online Figure V).

Measurements of Vascular Function. Microdissected renal interlobar arteries were mounted on wires in the chambers of a multivessel myograph (JP Trading, Aarhus, Denmark) filled with Krebs buffer (37°C) gassed with 95% O₂ / 5% CO₂. For 20-APheDa studies Sprague-Dawley (SD) rats were used. After mounting and 30-60 min of equilibration, the vessels were set to an internal circumference equivalent to 90% of that which they would have in vitro when placed under a transmural pressure of 100 mmHg. Isometric tension was monitored continuously before and after the experimental interventions. A cumulative concentration-response curve to phenylephrine (1x10⁻⁹-1x10⁻⁴ mol/L) was constructed. Additional experiments were conducted to determine concentration-response curves for acetylcholine-induced relaxation after maximal contraction. A cumulative relaxation-response curve to acetylcholine (1x10⁻⁸-1x10⁻⁴ mol/L) was constructed and the maximal relaxation was recorded. Wire myograph was used to measure constrictor responses to phenylephrine (10⁻⁹ to 5x10⁻⁶ mol/L) and relaxation to acetylcholine (10⁻⁹ to 5x10⁻⁶ mol/L) of renal interlobar arteries (~100 µm diameter).

Measurements of Media Thickness, Media to Lumen Ratio and Medial Cross-Sectional Area. Assessment of remodeling was performed by pressure myography according Schiffrin & Hayoz ¹³ and as previously described.¹⁴ Briefly, segments of renal interlobar arteries were dissected and mounted on a pressurized myograph and equilibrated for 1 h in oxygenated Ca²⁺-
free Krebs buffer at 37°C. The operator was blinded to treatments except for the blood pressure range of the animal. Lumen diameters from normotensive animals were determined at 100 mmHg and hypertensive animals at 140 mmHg. Measurements of outer diameter (OD) and inner diameter (ID) under passive conditions were used to calculate media thickness \([(OD – ID)/2\], media to lumen ratio \((OD – ID)/ID\)], and medial cross-sectional area \([CSA = (\pi/4) \times (OD^2 – ID^2)]\).

**Statistical Analysis** Data are expressed as mean ± SEM. Significance of difference in mean values was determined using t test and 1-way ANOVA, followed by the Newman-Keul post hoc test. P<0.05 was considered to be significant.

**REFERENCES**


FIGURE LEGENDS

**Figure I:** Schematic depicting the click-chemistry, retrieved proteomics data and target identification. The protein peptide summary represents the most frequent hits from proteomics analyses of six separate in-gel 20-APheDa-EC complex. The line between the marker lane (M) and the ECm lane reflects the fact that although the lanes are part of the same gel they are on a separate infrared channel.

**Figure II:** Tissue distribution of *Gpr75* expression in C57Bl/6 mouse tissues. cDNAs synthesized from a panel of various C57Bl/6 mouse tissues and Gpr75 mRNA levels were quantified by SYBR Green qPCR (A) and by TaqMan (B) using specific Gpr75 primers. Results are mean±SD, n=6 for all tissues except ovary, testis and uterus, n=3. Samples were run in triplicate.

**Figure III:** A representative immunoblot showing a single GPR75 immunoreactive band of ~55 kDa in EC lysate using the anti-GPR75 polyclonal antibody.

**Figure IV:** Effect of CCL5 on a) GPR75-G$_{\alpha_q/11}$ association, b) EGFR phosphorylation and c) ACE mRNA in EC. EC were incubated with CCL5 (1 nmol/L) for 5 min and cells were harvested and processed for immunoprecipitation of either GPR75 or EGFR followed by immunoblotting for (A) G$_{\alpha_q/11}$ and (B) phospho-tyrosine. For ACE mRNA (C), cells were incubated with CCL5 (1 nmol/L) for 2 h and processed for real-time PCR analysis of ACE mRNA. Results are mean±SEM, n=3.

**Figure V:** Representative radio-telemetric blood pressure measurements in a Cyp4a12tg mouse treated with either control or GPR75 shRNA and receiving DOX (n=1/group). A) Mean Arterial Pressure, B) Systolic Blood Pressure, C) Diastolic Blood Pressure and D) Heart Rate.

**Figure VI:** GPR75 protein levels in the liver (A) and heart (B) of mice receiving a bolus injection of SMARTvector Control shRNA or SMARTvector GPR75 shRNA (retro-orbital injection of 100 µl of 4.33x10$^9$ TU/mL of non-targeting or 7.29x10$^9$ TU/mL of GPR75-targeted shRNA particles, respectively). Tissues were harvested 35 days after injection and processed for measurements of GPR75 levels by Western blot (n=3; mean±SEM; *p<0.05)
**Click Chemistry**

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**Protein Peptide Summary**

- Ankyrin
- HIC-5
- Zinc Finger Protein
- Plasminogen-like
- Protein A
- Hyaluronan Synthase
- GTP-Binding Protein
- Vimentin
- Keratin
- Actin

**Interactome Tracing**

- HIC-5
- GIT1

**Orphan Receptor Protein Partner Analysis**

- Orphan Receptor Candidate

**Target Protein Confirmation**

- siRNA/shRNA

**On line Figure I**
(SYBR Green)

Relative Expression to Gapdh

Tissue

Aorta
Brain
Duodenum
Esophagus
Eye
Heart
Jejunum
Kidney
Large Intestine
Liver
Lung
Lymph Node
Ovary
Skeletal Muscle
Skin
Spleen
Stomach
Testis
Tongue
Uterus
Visceral rat

(TaqMan)

Relative Expression to Gapdh

Tissue

Aorta
Brain
Duodenum
Esophagus
Eye
Heart
Jejunum
Kidney
Large Intestine
Liver
Lung
Lymph Node
Ovary
Skeletal Muscle
Skin
Spleen
Stomach
Testis
Tongue
Uterus
Visceral rat
IP: GPR75

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IB: GPR75

GPR75
A

IP: GPR75

\( \Gamma_{\alpha_{q/11}} \)

\( \text{GPR75} \)

\( \text{Vehicle} \)

\( \text{CCL5} \)

\( -43 \)

\( -55 \)

B

IP: EGFR

\( \text{P-Tyr} \)

\( \text{EGFR} \)

\( \text{Vehicle} \)

\( \text{CCL5} \)

\( -170 \)

\( -170 \)

C

ACE mRNA

\( \text{Vehicle} \)

\( \text{CCL5} \)
A

Control shRNA  |  GPR75 shRNA
---|---
GPR75 |  
β-Actin |  

B

Control shRNA  |  GPR75 shRNA
---|---
GPR75 |  
α-tubulin |  

On line Figure VI

- 43  - 55  - 55  - 55

GPR75 / β-Actin (relative to control)

GPR75 / α-tubulin (relative to control)

*