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

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Abstract

Rationale—20-Hydroxyeicosatetraenoic acid (20-HETE), one of the principle cytochrome P450 (CYP) 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 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 $G\alpha_{q/11}$ protein dissociation and increased inositol phosphate (IP-1) accumulation as well as GPCR-kinase interacting protein-1 (GIT1)-GPR75 binding, which further facilitated the c-Src-mediated transactivation of endothelial EGFR. This results in downstream signaling pathways which induce angiotensin-converting enzyme (ACE) expression and endothelial dysfunction. Knockdown of GPR75 or GIT1 prevented 20-HETE-mediated endothelial growth factor receptor (EGFR) phosphorylation and ACE induction. In vascular smooth muscle cells, GPR75-20-HETE pairing is associated with $G\alpha_{q/11}$- and GIT1-mediated protein kinase C (PKC)-stimulated phosphorylation of MaxiKβ, λinking GPR75 activation to 20-HETE-mediated vasoconstriction.

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DISCLOSURES

None.

GPR75 knockdown in a mouse model of 20-HETE-dependent hypertension prevented blood pressure elevation and 20-HETE-mediated increases in ACE 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.

Keywords

20-HETE; GPR75; GIT1; EGFR; ACE; Vascular Remodeling; hypertension; cytochrome P450/ eicosanoids

Subject Terms

Vascular Biology; Hypertension; Cell Signaling/Signal Transduction; Remodeling

INTRODUCTION

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 etiology.[1] Studies from us and others, added 20-hydroxyeicosatetraenoic acid (20-HETE), the product of the ω-hydroxylation of arachidonic acid by cytochrome P450 (CYP) 4A and 4F isozymes,[2] to the list of factors contributing to the pathophysiology of hypertension and its cardiovascular consequences.[3–5] This is supported by studies identifying associations between variants in the human CYP4A11 and CYP4F2 genes and the prevalence of hypertension, myocardial infarction and stroke,[6–9] and clinical studies showing changes in plasma and urinary levels of 20-HETE in hypertension as well as in diseases and conditions such as cancer,[10] endothelial dysfunction,[11] oxidative stress,[12] obesity and metabolic syndrome,[13, 14] diabetes,[15] autosomal dominant polycystic kidney disease[16] and chronic kidney disease.[17, 18]

The proposed contributions of 20-HETE to the etiology 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 dysfunction and inflammation.[19, 20] 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 MAPK-IKKβ-NFκB transduction pathway leading to endothelial nitric oxide synthase (eNOS) uncoupling, inflammatory cytokine production and to increases in angiotensin-converting enzyme (ACE) expression and activity.[21–24] Though the mechanisms by which 20-HETE activates EGFR phosphorylation remained unknown, we postulated that 20-HETE could either: a) cross the cell membrane and stimulate tyrosine

kinases within the intracellular milieu, or b) 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 (Ang II).[25, 26] Interestingly, Akbulut *et al.*,[27] 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.[22, 28] With this in mind, we embarked on the challenging task of searching for the "20-HETE Receptor". Using crosslinking analogs, click 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 endothelial cells (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 endothelial cell 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 54-55 KDa protein band. 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 a tetracycline (doxycycline, DOX)-sensitive promoter have been previously described.[29] Male Cyp4a12tg mice (8–14-week-old) were used in all experiments. Mice were maintained on a 12 h light/dark cycle and fed *ad libitum*. DOX (1 mg/ml) was administered in the drinking water. In some experiments, Cyp4a12tg mice were anesthetized under isoflurane and administered lentiviral constructs via a single retro-orbital injection to maximize systemic distribution. Each mouse was injected with 100 μl of either 4.33×10^9 TU/mL of non-targeting or 7.29×10^9 TU/mL of GPR75-targeted shRNA particles. The administration of DOX (1 mg/ml) was initiated 48 h after the lentiviral injection. Blood pressure was monitored by radiotelemetry and tail cuff before and after administration of DOX for 14–35 days. At the end of all experiments, mice were anesthetized and laporotomy was performed. Renal preglomerular arteries (the whole circulatory tree) or interlobar arteries (80–100μm) were microdissected and collected for western blot analysis and functional studies. All results 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.

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*)-hydroxyeicosa-5(*Z*),14(*Z*)-dienamide (20-APheDa) (Fig. 1A), that contained both benzophenone, a photoreactive crosslinker for protein binding, and an azide, for selective binding and labeling to a click-chemistry dibenzocyclooctyne (DBCO) 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 (Fig. 1B). Incubation of 10 nmol/L of 20-APheDa with membrane fractions of human endothelial cells (EC) (20μg) followed by 15 min of UV (365 nm) crosslinking and 1h of incubation with the click reagent (DBCO-IRdye 800CW, LiCor) (50 μmol/L) yielded several bands, including a dominant band located at 47–49 kDa were detected (Fig. 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.[30] Nevertheless, the labeling of these bands by 20-APheDa (0.1 nmol/L) was competed by excess amounts of 20-HETE (100 μmol/L), but not 12-HETE (100 μmol/L) (Fig. 1D–E).

In-gel 20-APheDa-protein complexes were extracted from independent incubations and protein profiling 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 transforming growth factor beta-1-induced transcript 1 or hydrogen peroxide-inducible clone 5 (TGFB1I1/HIC-5),[31] POTE ankyrin domain family members, Zinc finger proteins, and GTP-binding proteins as top hits (Online Figure I). Further analysis also suggested domains and proteins associated with GIT1, a GPCR-kinase interacting protein-1 scaffold protein with ADP-ribosylating factor GTPase activity providing clues towards the possibility of a multi-protein receptor complex in close proximity to 20-APheDa's binding site.[32] With the identification of these two particular proteins we utilized protein partner analysis against orphan Gq Class A receptors referencing STRING and NCBI protein partner databases. The analysis revealed an association of HIC-5 and GIT1 with a candidate orphan receptor GPR75. To this end, in one out of three separate experiments GPR75-specific sequences were detected with C.I. 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 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 [$^3H_8$] 20-HETE (6.67 nmol/L) from EC membranes in a concentration-dependent manner with a calculated K_d of 3.75 nmol/L (Fig. 2B). Knockdown of GPR75 (Fig. 2C) prevented ^{3}H-20-HETE binding to EC membranes (Fig. 2D). Likewise, addition of the 20-HETE antagonist 20-5,16-HEDGE inhibited ^{3}H-20-HETE binding to EC membranes (Fig. 2F). This experiment confirmed our initial target protein identification (Online Figure I). Furthermore, the Kd values in these experiments (Fig 2B and E) accurately represent 20-HETE's biological activities wherein maximal changes in nitric oxide (NO) bioavailability and ACE expression have been seen in the range of 1–10 nmol/L.[22–24] Since GPR75 is a $G\alpha_q$-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 min increased IP-1 accumulation by 4-fold; this increase was not observed in GPR75-knockdowned EC (Fig 2G–H). 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 $G\alpha_{q/11}$, GIT1 and HIC-5

Antibodies against the GPR75 c-terminal, which recognize a single 54-55 KDa protein band (Online Figure III), were used in all subsequent experiments. A representative image of kidney sections co-stained with antibodies against GPR75 and CD31, an endothelial cell marker, showed a clear co-localization of GPR75 to the vascular endothelium and expression of GPR75 can be observed throughout the vessel (Fig. 3A). Moreover, immunoprecipitation and immunoblotting experiments indicated that $G\alpha_{q/11}$ as well as G Protein-Coupled Receptor Kinase Interactor 1 (GIT1) and Hydrogen Peroxide-Inducible Clone 5 (HIC-5) are associated with GPR75 in EC (Fig. 3B).

20-HETE alters the association of GPR75 with $G\alpha_{q/11}$, GIT1 and HIC-5 in HMVEC

Incubation of EC with 20-HETE (5 min) decreased GPR75-$G\alpha_{q/11}$ association by 44 and 51% at 1 and 10 nmol/L (Fig. 3C). In addition, 20-HETE also increased GPR75-GIT1 binding by 2-fold and HIC-5-GPR75 dissociation by 60% (Fig. 3D–E). Importantly, 20-6,15-HEDGE, an effective 20-HETE antagonist in vitro and in vivo,[29, 33] did not increase GIT1-GPR75 association. Moreover, it prevented 20-HETE-stimulated GIT1-GPR75 association (Fig. 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 MAPK-IKKβ-NFκB signaling leading to eNOS uncoupling, ACE induction and inflammatory cytokine production in EC.[22, 24, 34] 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 (Fig. 4A–B). As previously shown,[24] 20-HETE increases total tyrosine phosphorylation of immunoprecipitated EGFR in EC transduced with control siRNA by 1.99±0.16-fold (Fig.

4C). 20-HETE-stimulated EGFR tyrosine phosphorylation was completely prevented in EC transduced with either GPR75 or GIT1 siRNA (Fig. 4C). In addition, within the same time frame in which 20-HETE stimulated a 2.3-fold increase in EGFR phosphorylation (Fig. 4D), it also decreased the association of c-Src with GIT1 by 72% (Fig. 4E) and increased association of c-Src with EGFR by 110% (Fig. 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 (Fig. 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 $G\alpha_{q/11}$-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 (DOX) display, upon administration of DOX, 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.[29, 33] We used this model to assess whether GPR75 is necessary for the pro-hypertensive 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 DOX in the drinking water to induce Cyp4a12-20-HETE synthase. As expected, administration of DOX 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 mmHg, respectively) (Fig. 5A–B). In contrast, DOX administration to Cyp4a12tg mice that received a bolus of GPR75-targeted shRNA failed to increase blood pressure (110±2 mmHg) (tail cuff, Fig. 5A–B & 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 (Fig. 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 DOX-induced 20-HETE-dependent hypertension in Cyp4a12tg mice.[33] In line with previous studies, vascular ACE expression in DOX- and DOX+control shRNA-treated Cyp4a12tg mice increased by 3.3- and 3.8-fold, respectively (Fig. 5D). In contrast, vascular ACE expression was not induced in DOX-treated Cyp4a12tg mice that received GPR75-targeted shRNA lentiviral particles (Fig. 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 DOX-treated Cyp4a12tg mice is associated with 20-HETE-dependent endothelial dysfunction and enhanced sensitivity to constrictor

stimuli.[29, 33] Here we show that knockdown of GPR75 interferes with the ability of DOX to impair relaxations to acetylcholine and increase contractions to phenylephrine. The relaxation to acetylcholine was markedly reduced in interlobar arteries from DOX-treated (55%±3%) as compared to arteries from water-treated Cyp4a12tg mice (99±2%). Administration of GPR75-targeted, but not control, shRNA lentiviral particles prevented DOX-induced impairment in relaxation to acetylcholine (88%±3%) (Fig. 6A). Likewise, administration of GPR75-targeted shRNA prevented the DOX-induced increases in contractions to phenylephrine. Treatment with DOX increased sensitivity to phenylephrine ($p<0.05$) as evidenced by a reduction in EC_{50} (from 0.75±011 to 0.40±0.08 μmol/L) and an increase in R_{max} (from 4.89±0.51 to 6.17±0.39 mN/mm) when compared to water-treated Cyp4a12tg mice (Fig. 6B). However, the EC_{50} to phenylephrine in arteries from DOX-treated mice that received GPR75-targeted shRNA was unchanged (0.86±0.24 μmol/L) and was not different from the EC_{50} in arteries from control mice (Fig. 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 DOX leads to hypertrophic remodeling of the renal microvessels in a 20-HETE-dependent manner that is largely independent of blood pressure elevation.[33, 36] In this study, assessment of remodeling in interlobar arteries (~80-100 μm) showed that hypertension in Cyp4a12tg mice receiving control shRNA+DOX for 35 days was associated with a 3-fold increase in media thickness, media to lumen ratio and cross sectional area (Fig. 6C–F). In contrast, neither hypertension nor microvascular remodeling occurred in arteries from DOX-treated Cyp4a12tg mice that received GPR75-targeted shRNA (Fig. 6C–F). 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 $G\alpha_{q/11}$ and GIT1-mediated signaling

20-HETE has been shown to stimulate vascular smooth muscle contraction via mechanisms that include activation of PKCα, MAPK and Src-type tyrosine kinases, all of which can phosphorylate and inhibit the Ca^{2+}-activated K^+ channels (BK_{ca}), leading to depolarization, elevation in cytosolic [Ca^{2+}], and increased Ca^{2+} entry through the L-type Ca^{2+} channels.[37] As seen in Figure 7, GPR75 is expressed in cultured aortic vascular smooth muscle cells. In these cells, 20-HETE stimulated the dissociation of $G\alpha_{q/11}$ from GPR75 (Fig. 7A), GPR75-GIT1 association (Fig. 7B), GIT1-PKCα dissociation (Fig. 7C), PKCα-MaxiKβ association (Fig. 7D), c-Src-MaxiKβ association (Fig. 7E) and MaxiKβ tyrosine phosphorylation (Fig. 7F). The phosphorylation of the MaxiKβ subunit of the BK_{ca} channels leads to inactivation of the channel and consequently to vasoconstriction.[38]

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.[5] 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 cytochrome P450-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. To overcome some of these difficulties, we employed 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 towards identifying receptor candidates. Click-chemistry compounds have been proven to be useful for labeling and used for the identification of multi-protein 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 $G\alpha_{q/11}$-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 $G\alpha_{q/11}$ 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 al.*,[39] 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 al.*, reported that the chemokine RANTES (CCL5) increased IP_3 and intracellular Ca^{2+} in CHO or HEK cells overexpressing the mouse GPR75 via a Gq protein-coupled PLC-mediated signal transduction.[35] No direct binding studies were documented in that study. A recent study by Liu et al. showed that CCL5 stimulates insulin secretion in isolated islets via PLC-activated Ca^{2+} influx in a GPR75-dependent manner; however, they also did not document direct binding or interaction between GPR75

and CCL5.[41] 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 (NC-IUPHAR) still classifies GPR75 as an orphan receptor.[42, 43] Notably, in our hands, CCL5 failed to dissociate $G\alpha_{q/11}$ from GPR75 in EC. Moreover, CCL5 lacked the ability to transactivate EGFR and induce ACE mRNA, two 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 two key original findings. First, when applied to EC, 20-HETE binds and activates the receptor at a concentration which elicits a specific EGFR-MAPK-IKK-NFkB signaling pathway to induce ACE transcription, uncouple eNOS and stimulate cytokine production.[22, 24, 34, 44] This activation involves dissociation of $G\alpha_{q/11}$ from GPR75 and increased GIT1-GPR75 association following 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 (Fig. 8). The dissociation of $G\alpha_{q/11}$ from GPR75 following addition of 20-HETE, most likely, initiated a PLC-IP3 mediated increases in $[Ca^{2+}]$, 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 BK_{ca} channels underlies 20-HETE-mediated vasoconstriction.[45] Here we link GPR75-20-HETE pairing to stimulation of $G\alpha_{q/11}$ dissociation and GIT1-mediated PKCα- and cSrc-stimulated tyrosine phosphorylation of MaxiKβ which inactivates the channel[38, 46] and alters BK_{ca} subunit trafficking[47] leading to cell depolarization, elevation in cytosolic $[Ca^{2+}]$ 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[48] and co-activate the androgen receptor.[49] The interaction between HIC-5 and the androgen receptor may account for the strong relationship between androgens and 20-HETE.[50]

The second key finding in this study is that the expression of GPR75 is required for the 20-HETE pro-hypertensive activities. The conditional Cyp4a12tg mice display DOX-mediated hypertension along with vascular dysfunction and remodeling in a 20-HETE-dependent manner.[29, 33] 20-HETE-mediated vascular dysfunction and remodeling has been shown to be largely independent of blood pressure elevation.[33, 36] We hypothesized that if GPR75 is the 20-HETE receptor, its disruption should prevent hypertension in the DOX-treated Cyp4a12tg mice. Indeed, knockdown of GPR75 in DOX-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 anti-hypertensive 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 II-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 pro-hypertensive mechanisms via vascular and tubular actions that foster vasoconstriction and/or conservation of sodium, and to anti-hypertensive 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 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, myocardial infarction) while genetic studies of receptor variants could lead to a better understanding of some discrepancies between 20-HETE levels and reported pro- or anti-hypertensive properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

20-HETE	20-hydroxyeicosatetraenoic acid
CYP	Cytochrome P450
EGFR	Epidermal Growth Factor Receptor
eNOS	Endothelial Nitric Oxide Synthase
IKKβ	Inhibitor of Nuclear Factor Kappa-B Kinase Subunit B*eta*
NFκB	Nuclear Factor Kappa B
MAPK	Mitogen activated protein kinase
ACE	Angiotensin Converting Enzyme
Ang II	Angiotensin II
GPCR	G-Protein Coupled Receptor
GPR75	G-Protein Receptor 75
20-APheDa	20-azido-N-((4-(3-(4-benzoylphenyl)propanamido)-phenyl)sulfonyl)-19(*S*)-hydroxyeicosa-5(*Z*), 14(*Z*)-dienamide
12-HETE	12-Hydroxyeicosatetraenoic Acid
20-HEDE	20-hydroxyeicosa-6(*Z*), 15(*Z*)-dienoic acid
DBCO	Dibenzocyclooctyne
TGFB1I1	Transforming Growth Factor Beta-1-Induced Transcript 1
HIC-5	Hydrogen Peroxide-Inducible Clone 5
GIT1	G Protein-Coupled Receptor Kinase Interactor 1
NO	Nitric Oxide
IP-1	Inositol Phosphate
PKC	Protein Kinase C
DOX	Doxycycline
MaxiKβ	Large Conductance Voltage and Calcium-Activated Potassium Subunit Betaα

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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.
- The actions of 20-HETE appear 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 GPR75, an orphan G-protein-coupled receptor (GPCR), as the putative 20-HETE-receptor. This is the first time a GPCR 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 vascular ACE expression, endothelial dysfunction, contractility, remodeling and hypertension.
- The discovery of 20-HETE-GPR75 pairing opens the door for future research with regards to how this lipid-receptor interaction is involved in a variety of pathologies known to be closely mediated and associated with 20-HETE.

The high prevalence and the significant contribution of hypertension to cardiovascular and renal disease comprise its position as the top contributor to the burden of disease worldwide. However, with few exceptions, the molecular basis underlying the pathogenesis of hypertension remains to be defined. Moreover, despite the large number of available treatment options, a significant portion of the hypertensive population has uncontrolled blood pressure. The plethora of available anti-hypertensive drugs, while lowering blood pressure, do not always correct the organ damage associated with hypertension. 20-HETE has been identified as a significant contributing factor to the pathophysiology of hypertension and its cardiovascular consequences. In this study, we identified a cell surface receptor, the orphan GPCR (Gq) GPR75, to which 20-HETE binds to and initiates a cell-signaling cascade leading to endothelial cell dysfunction and vascular smooth muscle contractility. This study further show that knockdown of GPR75 prevents 20-HETE-mediated vascular remodeling and hypertension. The identification of 20-HETE-GPR75 pairing opens the door for discovery and there is much to be explored including the role of this interaction in a variety of diseases/pathologies associated with 20-HETE (e.g., stroke, myocardial infarction).

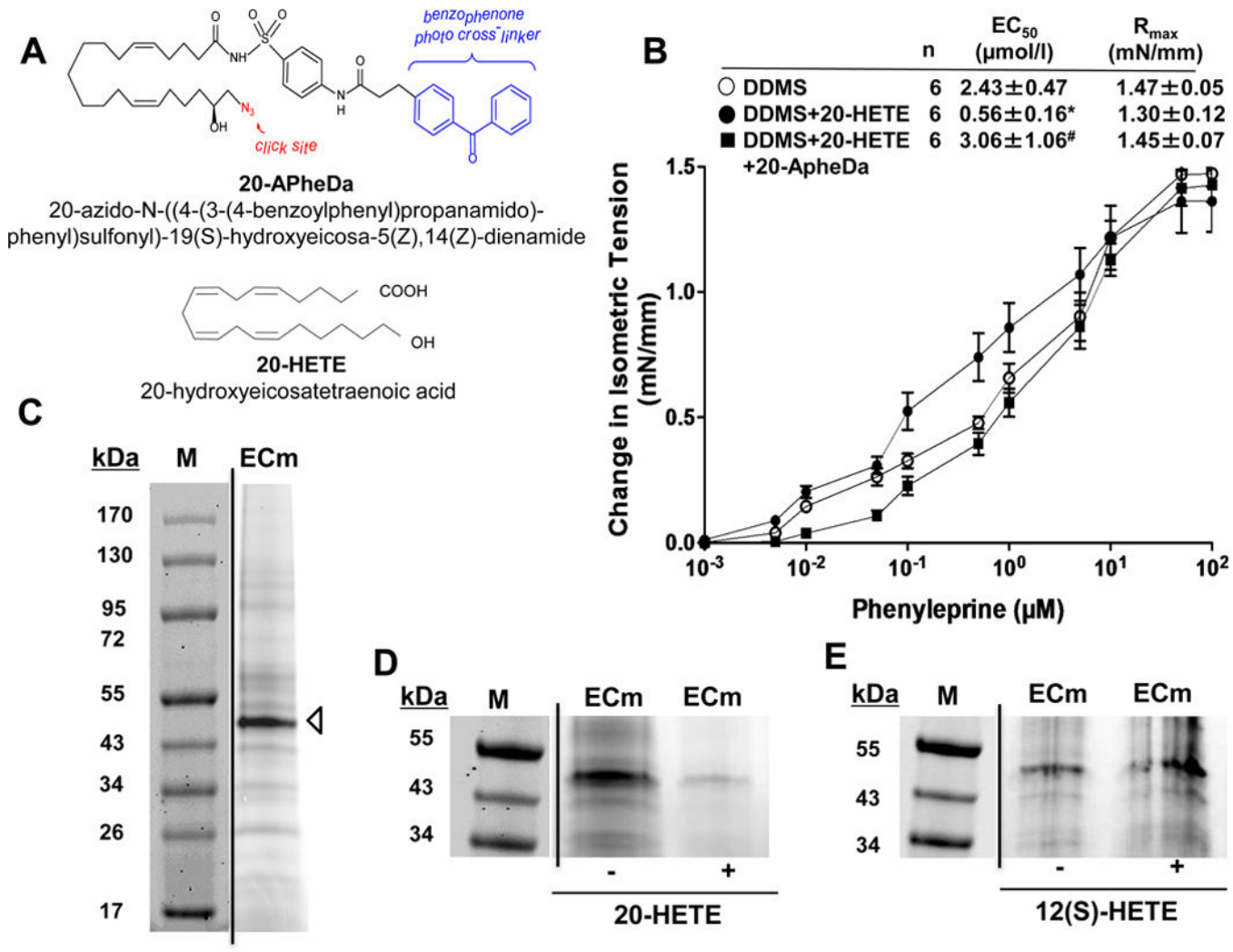

Figure 1. Isolation of a 20-HETE-associated membrane protein complex by the click-in chemistry

A) Structures of the pharmacological probe 20-azido-N-4-(3-(4-benzoylphenylpropanamido)phenylsulfonyl)-19(S)-hydroxyeicosa-5(Z),14(Z)-dienamide (20-APheDa) and 20-HETE. B) Cumulative concentration-response curve to phenylephrine in renal interlobar arteries from C57BL/6 mice preincubated with the 20-HETE biosynthesis inhibitor DDMS (30 µmol/L) and cumulative concentration responses to phenylephrine (10-3 to 102 µmol/L) was constructed in the presence and absence of 20-HETE (10 µmol/L) or 20-HETE (10 µmol/L) +20-APheDa (10 µmol/L) (*p<0.05 vs DDMS; 3p<0.05 vs DDMS +20-HETE; mean±SEM, n=6). C) A representative (n=8) In-gel image band composition of 20-APheDa (10 nmol/L) bound to human microvascular endothelial cell membrane proteins (ECm, 20µg). D–E) Binding of 20-APheDa (0.1 nmol/L) to ECm (10 µg) is competed for by 20-HETE (100 µmol/L) but not by 12(S)-HETE (100 µmol/L) (representative image, n=5-6). The line between the marker lane (M) and the ECm lane in panels C-E reflects the fact that although the lanes are part of the same gel they are on a separate infrared channel.

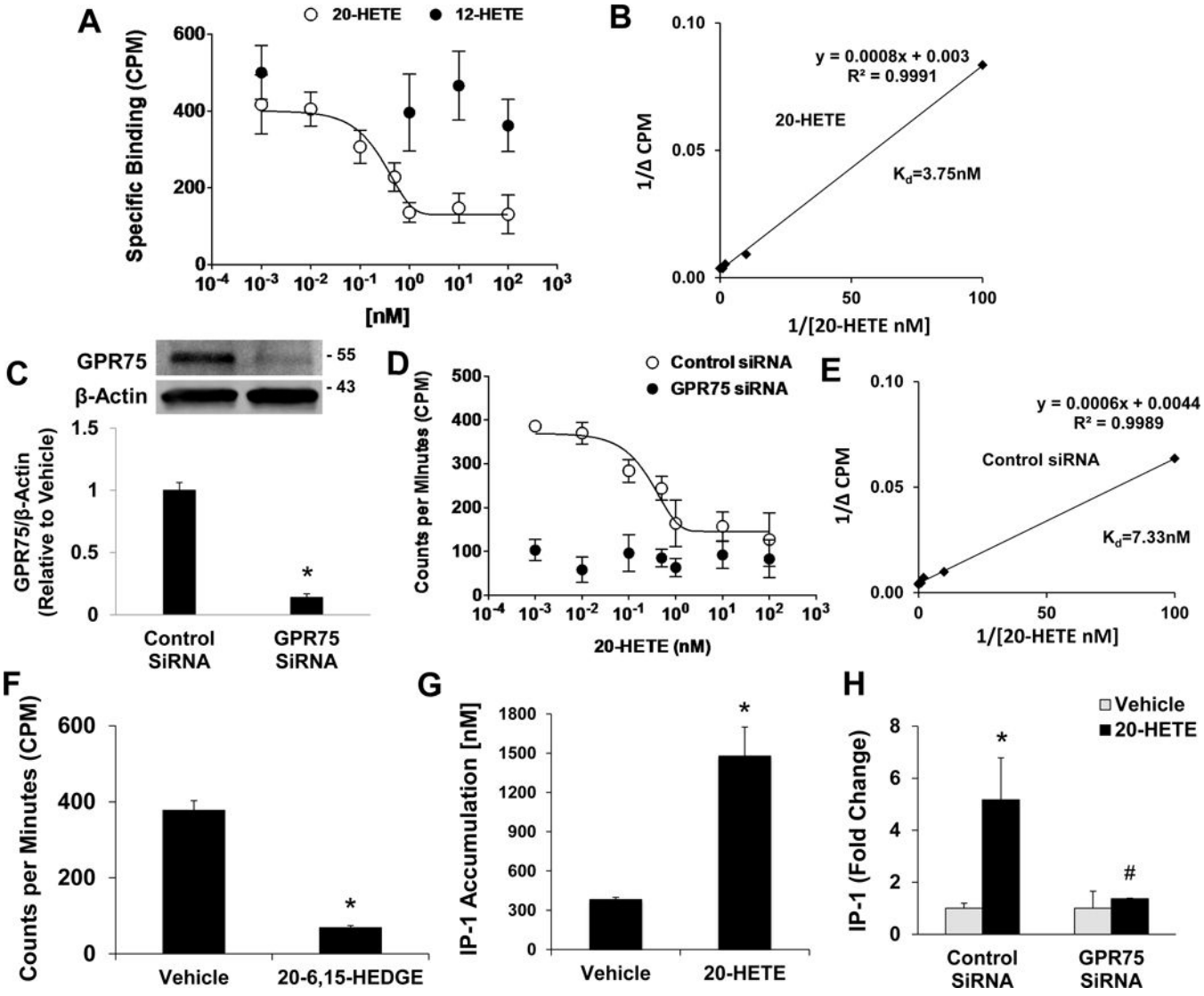

Figure 2. 20-HETE binds to GPR75

A) Displacement of radiolabeled [$^3H_8$] 20-HETE in membranes by unlabeled 20-HETE and 12(*S*)-HETE. **B)** Assessment of Kd. **C)** GPR75 protein levels in EC treated with control and GPR75-specific siRNA for 36 h. **D)** Displacement of radiolabeled [$^3H_8$] 20-HETE (6.67 nmol/L) by increasing concentrations of unlabeled 20-HETE in membranes from EC treated with control and GPR75-specific siRNA for 36 h. **E)** Assessment of Kd from displacement bindings in 3D. **F)** Displacement of [$^3H_8$] 20-HETE by 20-6,15-HEDGE (1 nmol/L). **G–H**) Effect of 20-HETE (10 nmol/L) on IP-1 accumulation in EC treated with control and GPR75-specific siRNAs. All results are mean±SEM (n=4–6, *p<0.05 vs. vehicle, $^{\#}$p<0.05 vs. 20-HETE)

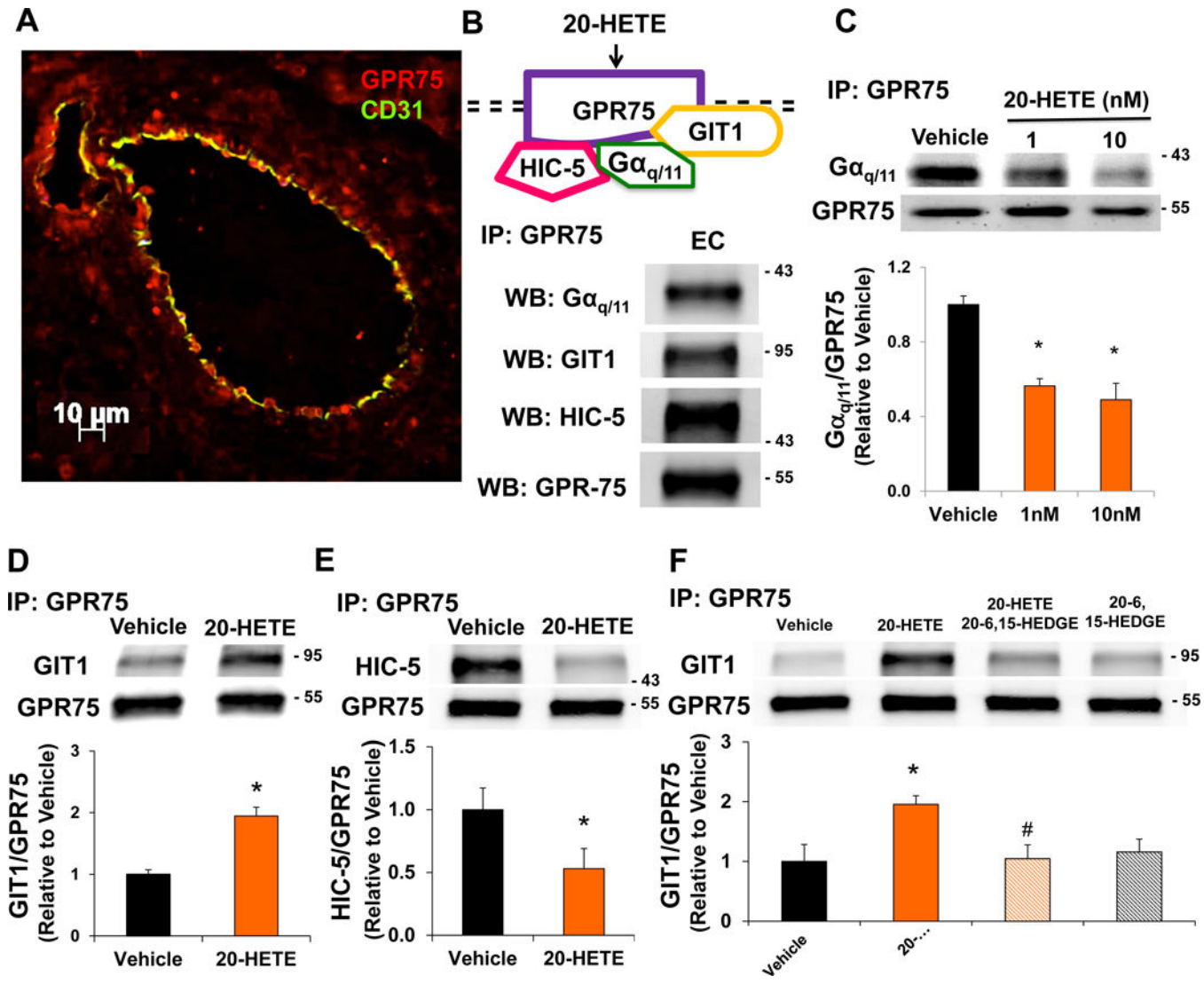

Figure 3. Expression of GPR75 in the vascular endothelium and effect of 20-HETE on GPR75 association with GIT1, HIC-5 and $G\alpha_{q/11}$ in EC

A) A representative image of GPR75 (red) and CD31 (green) immunofluorescence of kidney sections from C57BL/6 mice (20X; scale bar, 10μm). **B)** A representative Western blot of GPR75 immunoprecipitate showing association with $G\alpha_{q/11}$, GIT1 and HIC-5 in EC. 20-HETE alters GPR75 association with (**C**) $G\alpha_{q/11}$, (**D**) GIT1 and (**E**) HIC-5. **F)** Effect of 20-6,15-HEDGE on 20-HETE-stimulated GPR75-GIT1 association. EC were incubated with 20-HETE (1 or 10 nmol/L) with and without 20-6,15-HEDGE (10 nmol/L) for 5 min and GPR75 was immunoprecipitated and immunoblot for the indicated proteins (mean ±SEM, n=4, *p<0.05 vs Vehicle, #p<0.05 vs 20-HETE).

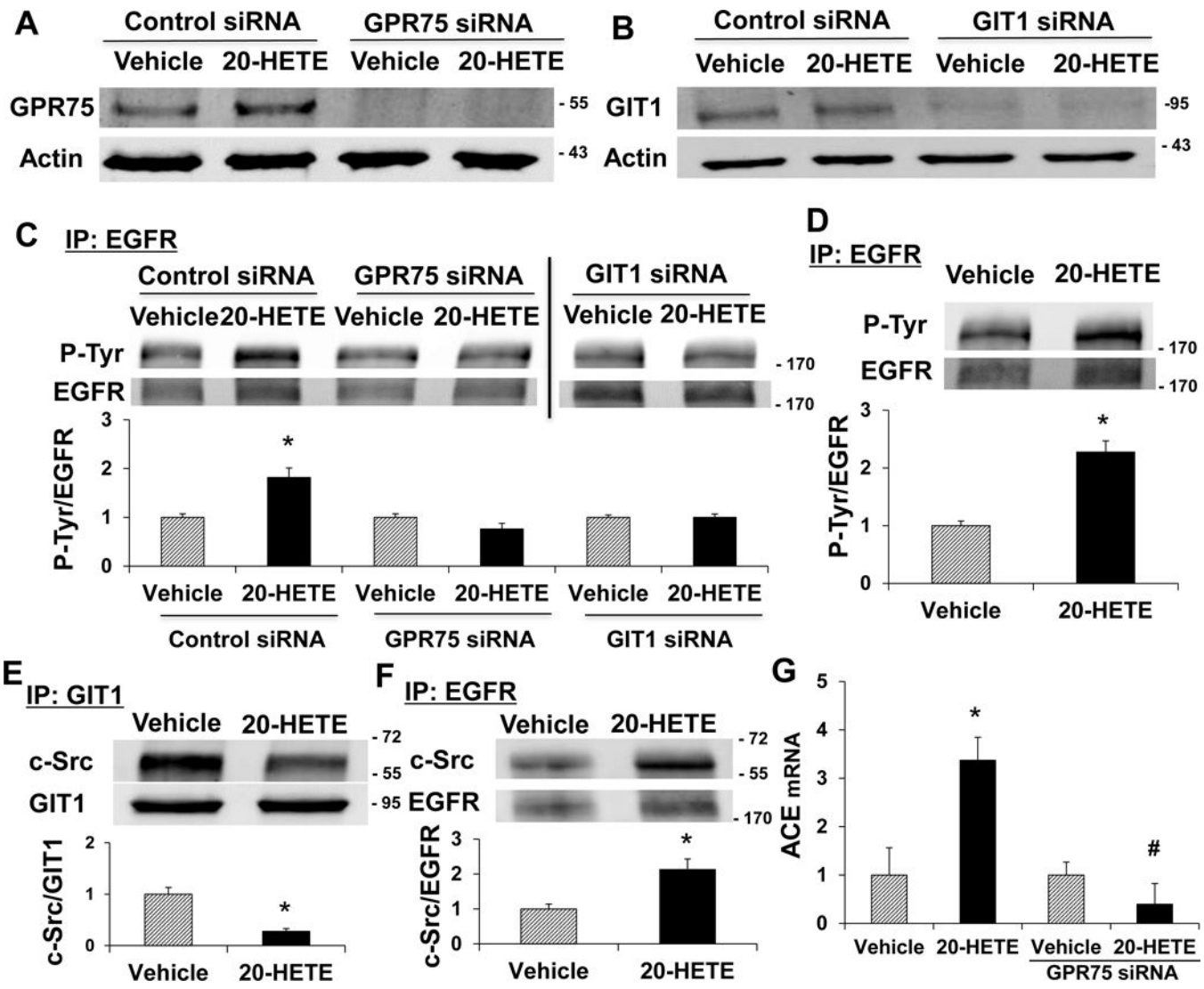

Figure 4. GPR75 and GIT1 are required for 20-HETE signaling in EC (A–C)

EC were transfected with control siRNA, GPR75- or GIT1-specific siRNA for 36 h. Cells were then incubated in a serum free media for 12 h prior to addition of 20-HETE (10 nmol/L) for 5 min. **A)** Representative western blot showing GPR75 and **B)** GIT1 knockdown. **C)** Effect of GPR75 and GIT1 knockdown on 20-HETE-stimulated EGFR phosphorylation (immunoblots are from separate experiments noted by dividing line; mean ±SEM, n=4, *p<0.05 vs. Vehicle). **20-HETE stimulation of EGFR phosphorylation is mediated by GPR75-GIT1-cSrc activation in EC (D–F)**. The effects of 20-HETE (10 nmol/L) treatment (5 min) on: **D)** phosphorylated EGFR, **E)** c-Src bound to GIT and **F)** c-Src bound to EGFR, (mean±SEM, n=4, *p<0.05 vs Vehicle). **G) GPR75 knockdown prevents 20-HETE-mediated ACE induction in EC**. Endothelial ACE mRNA expression from control and GPR75 siRNA treated cells in the presence and absence of 20-HETE (10 nmol/L) for 2 h (mean±SEM, n=3, *p<0.05 vs Vehicle, #p<0.05 vs 20-HETE).

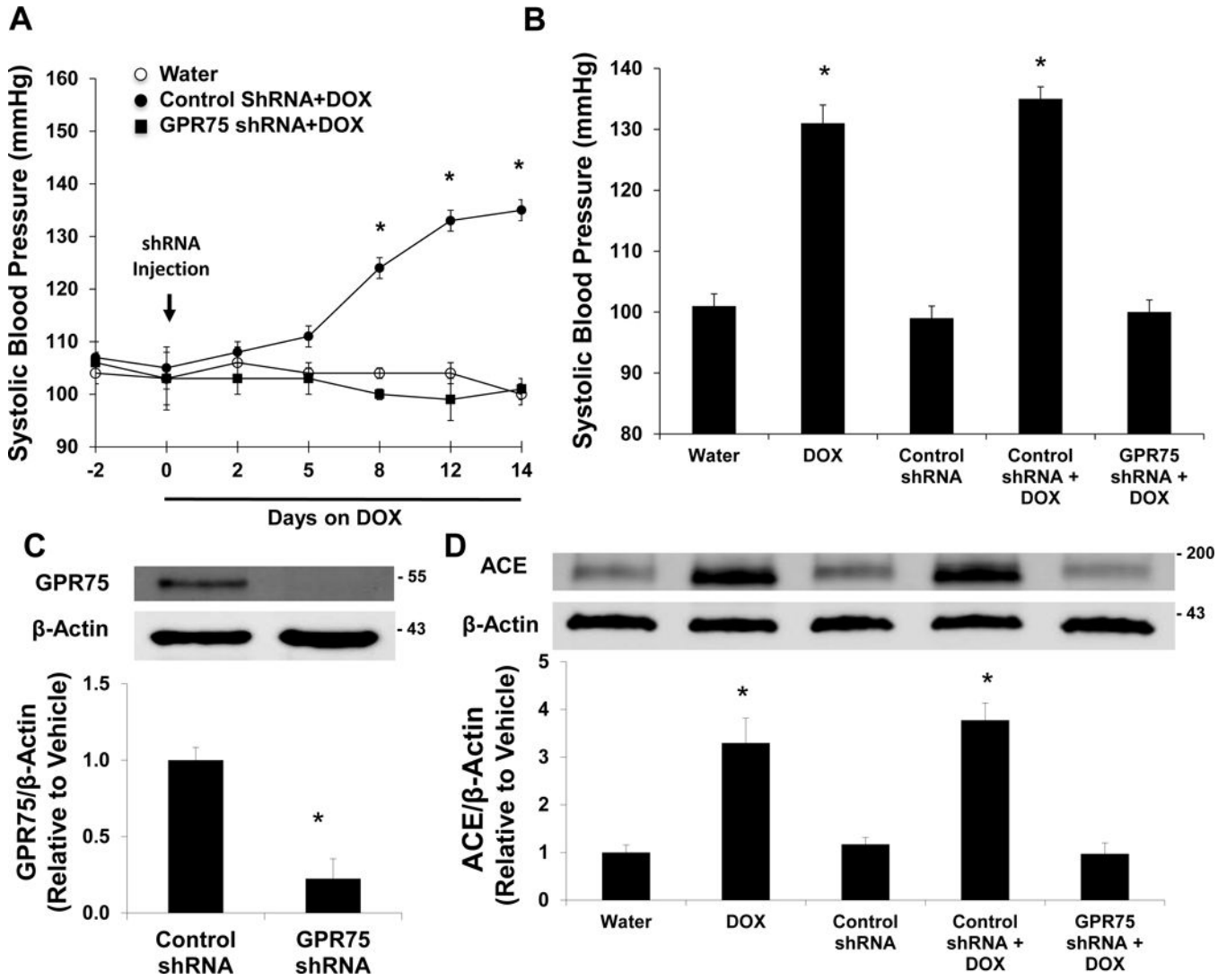

Figure 5. GPR75 Knockdown Prevents 20-HETE-Dependent Hypertension and ACE induction in Cyp4a12tg Mice

A) Systolic blood pressure monitoring in Cyp4a12tg mice receiving water, control shRNA +DOX and GPR75 shRNA+DOX (n=4, *p<0.05 vs water) for 14 days. **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 PGMVs 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 days. Blood pressure was measured by the tail cuff method. Results are mean±SEM, n=4, *p<0.05 vs water or control shRNA+DOX.

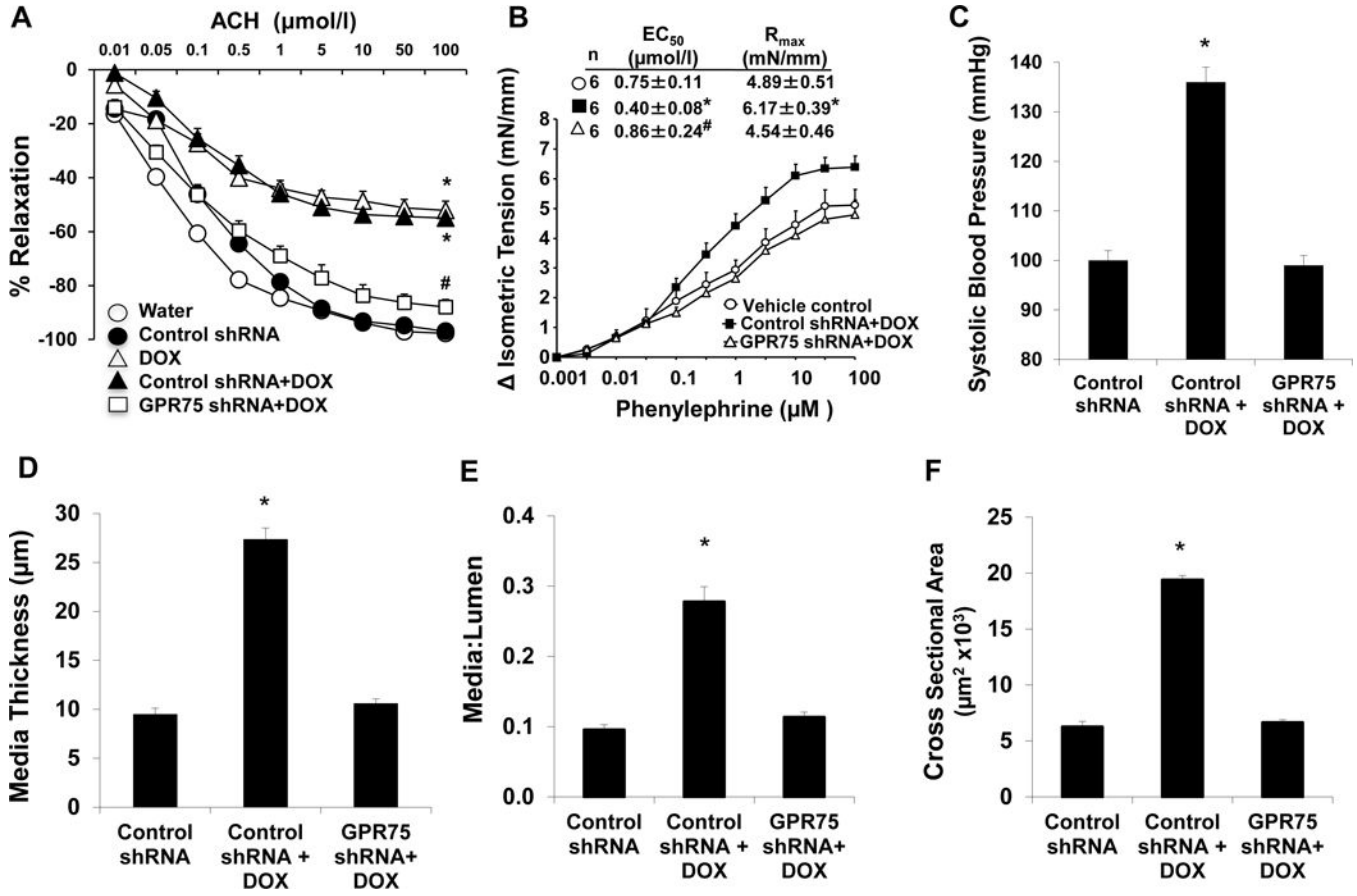

Figure 6. GPR75 knockdown prevent 20-HETE-dependent hypertension, vascular dysfunction and vascular remodeling (A–F)

A) Cumulative concentration-response curve to acetylcholine in renal interlobar arteries from Cyp4a12tg mice treated with water, Control shRNA, DOX, control shRNA+DOX, and GPR75 shRNA+DOX for 14 days (meaan±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 days. 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 days (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-to-lumen ratio and **F)** cross sectional area in renal interlobar arteries from mice treated with control and GPR75 shRNA after receiving DOX for 35 days as previously described[39] (n=4, *p<0.05 vs water, #p<0.05 vs control shRNA+DOX).

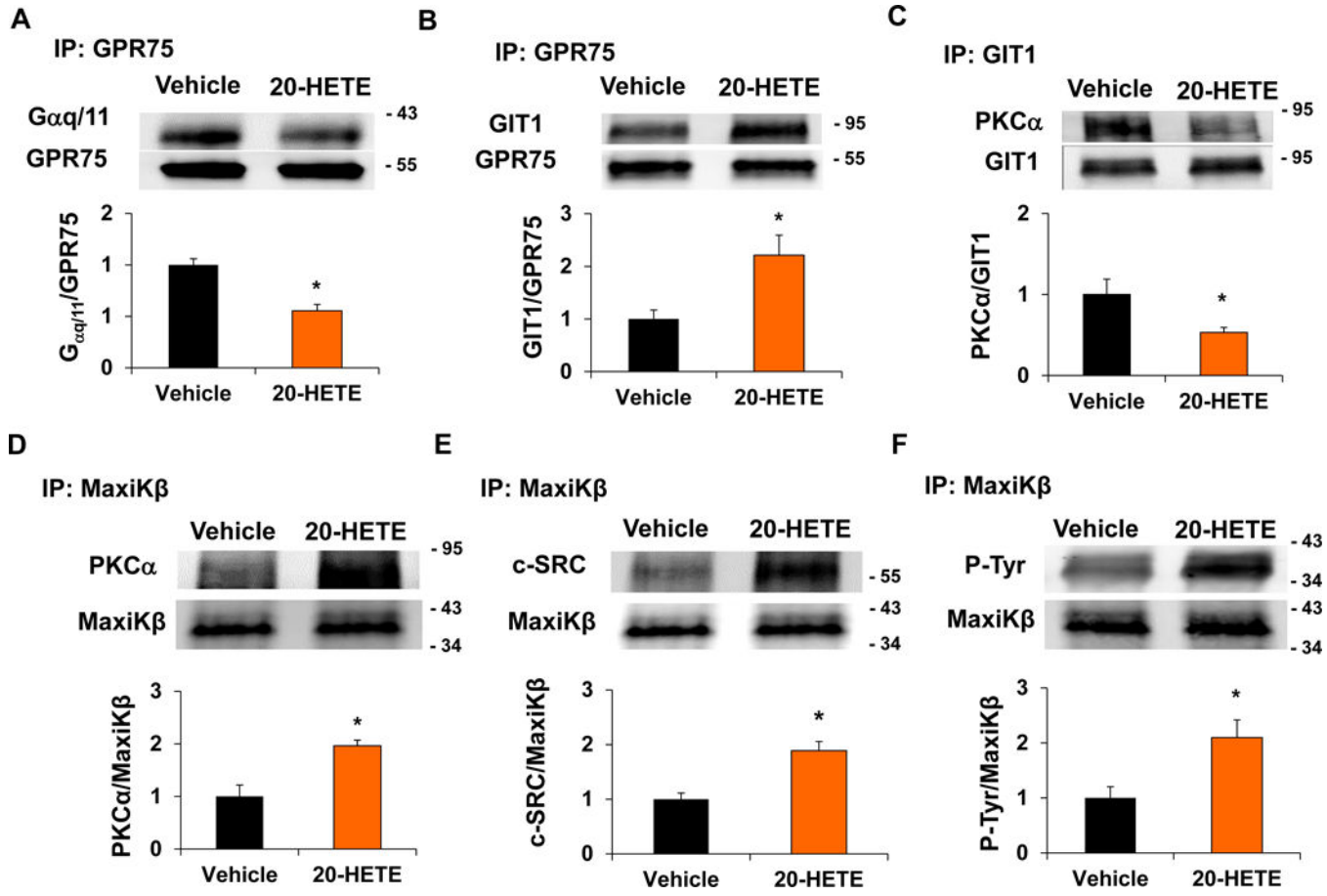

Figure 7. GPR75-20-HETE pairing in vascular smooth muscle is linked to inhibition of MaxiKβ

Cultured aortic vascular smooth muscle cells were treated with 20-HETE (10 nmol/L) for 5 min. Immunoprecipitation followed by immunoblotting and densitometry analysis showed increases in: **A**) $G\alpha_{q/11}$ dissociation from GPR75, **B**) GPR75-GIT1 association, **C**) GIT1-PKCα dissociation, **D**) PKCα-MaxiKβ association, **E)** cSrc-MaxiKβ association and **F**) MaxiKβ tyrosine phosphorylation (mean±SEM, n=4, *p<0.05 vs Vehicle).

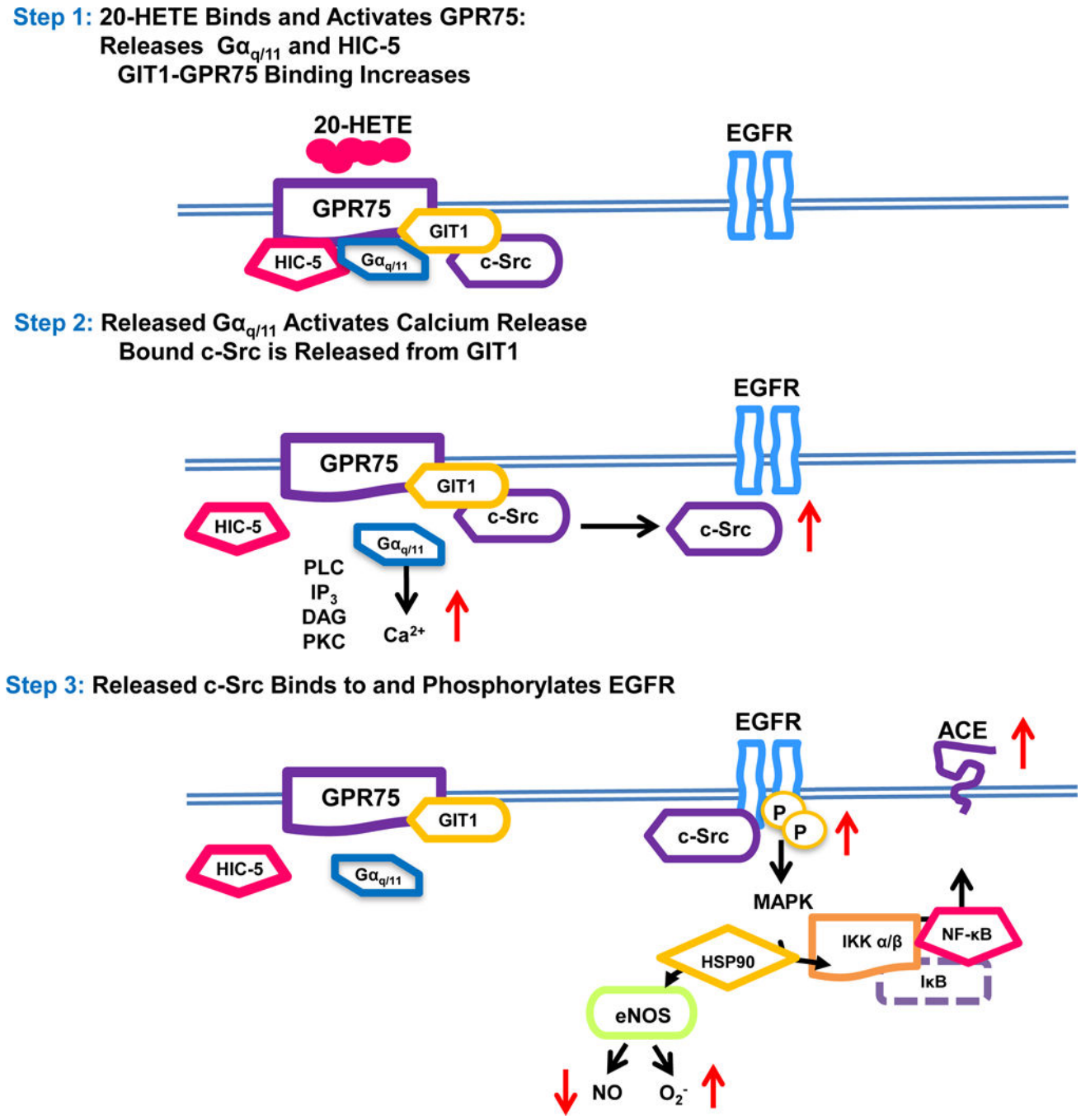

Figure 8. Proposed 20-HETE-GPR75-mediated signaling in endothelial cells

20-HETE-GPR75 pairing stimulates the dissociation of $G\alpha_{q/11}$ and the association of GIT1 to the receptor. The later facilitates c-Src-mediated EGFR transactivation. The 20-HETE-GPR75-mediated activation of EGFR results in the stimulation of downstream cascades that regulate vascular ACE expression and decreases in NO bioavailability.