Fatty Acid Receptor Gpr40 Mediates Neuromicrovascular Degeneration Induced by Transarachidonic Acids in Rodents

Jean-Claude Honoré,* Amna Kooli,* David Hamel,* Thierry Alquier, José-Carlos Rivera, Christiane Quiniou, Xin Hou, Elsa Kermorvant-Duchemin, Pierre Hardy, Vincent Poitout, Sylvain Chemtob

Objective—Nitro-oxidative stress exerts a significant role in the genesis of hypoxic-ischemic (HI) brain injury. We previously reported that the ω-6 long chain fatty acids, transarachidonic acids (TAAs), which are nitrative stress-induced nonenzymatically generated arachidonic acid derivatives, trigger selective microvascular endothelial cell death in neonatal neural tissue. The primary molecular target of TAAs remains unidentified. GPR40 is a G protein–coupled receptor activated by long chain fatty acids, including ω-6; it is highly expressed in brain, but its functions in this tissue are largely unknown. We hypothesized that TAAs play a significant role in neonatal HI-induced cerebral microvascular degeneration through GPR40 activation.

Approach and Results—Within 24 hours of a HI insult to postnatal day 7 rat pups, a cerebral infarct and a 40% decrease in cerebrovascular density was observed. These effects were associated with an increase in nitrative stress markers (3-nitrotyrosine immunoreactivity and TAA levels) and were reduced by treatment with nitric oxide synthase inhibitor. GPR40 was expressed in rat pup brain microvasculature. In vitro, in GPR40-expressing human embryonic kidney (HEK)-293 cells, $[^{14}$C]-14E-AA (radiolabeled TAA) bound specifically, and TAA induced calcium transients, extracellular signal–regulated kinase 1/2 phosphorylation, and proapoptotic thrombospondin-1 expression. In vivo, intracerebroventricular injection of TAAs triggered thrombospondin-1 expression and cerebral microvascular degeneration in wild-type mice, but not in GPR40-null congeners. Additionally, HI-induced neurovascular degeneration and cerebral infaract were decreased in GPR40-null mice.

Conclusions—GPR40 emerges as the first identified G protein–coupled receptor conveying actions of nonenzymatically generated nitro-oxidative products, specifically TAAs, and is involved in (neonatal) HI encephalopathy. (Arterioscler Thromb Vasc Biol. 2013;33:954-961.)

Key Words: endothelial cell death ■ fatty acids ■ GPR40 ■ neonatal hypoxia-ischemia ■ transarachidonic acids

A

Received on: December 4, 2012; final version accepted on: March 4, 2013.

From the Department of Pediatrics, Research Center - CHU Ste-Justine, Montréal, Québec, Canada (J.-C.H., A.K., D.H., J.-C.R., C.Q., X.H., E.K.-D., P.H., S.C.); Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada (A.K.); Department of Pharmacology, Université de Montréal, Québec, Canada (J.-C.R., D.H., J.-C.R., S.C.); Montreal Diabetes Research Center - CRCHUM, Montréal, Quebec, Canada (T.A., V.P.); Department of Medicine, Université de Montréal, Québec, Canada (T.A., V.P.); and Necker-Enfants malades Hospital, INSERM U872 and AP-HP, Paris Descartes University, Paris, France (E.K.D.).

*These authors contributed equally to this work.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBHA.112.300943/-/DC1.

Correspondence to Sylvain Chemtob, MD, PhD, Research Center - CHU Ste-Justine, Departments of Pediatrics, Ophthalmology, and Pharmacology, 3175, Chemin Côte Ste-Catherine, Montréal, Québec, Canada H3T 1C5. E-mail sylvain.chemtob@umontreal.ca

© 2013 American Heart Association, Inc.
GPR40 has been described as a receptor for long chain FAs, including ω-6,19–21 whereas GPR120, the other long chain FA receptor, is mostly activated by ω-3 long chain FAs.21 In humans, GPR40 is principally expressed in pancreatic islets and brain tissue.19 Numerous studies examined the role of GPR40 in the FA-induced potentiation of insulin secretion and in β-cell dysfunctions associated with type 2 diabetes mellitus, and evaluated the use of GPR40 agonists as potential therapeutic options for the treatment of type 2 diabetes mellitus.20,22,23 However, the role of GPR40 in brain specifically in therapeutic options for the treatment of type 2 diabetes mellitus, and evaluated the use of GPR40 agonists as potential and in β and brain tissue.19 Numerous studies examined the role of humans, GPR40 is principally expressed in pancreatic islets and brain sections did not reveal vascular degeneration, previously reported.7 As illustrated, sham, hypoxic alone, or ischemic newborn, we proceeded to identify the primary target of To elucidate the specific role of TAA in HI injury in the newborn, we proceeded to identify the primary target of TAA. Because GPR40 receptor is a putative receptor for long chain FAs19,20 and cis-AA and TAAs share very high structural homology,16,25 we determined whether TAA can bind and activate GPR40. GPR40 specifically localized on cerebral microvessels (Figure 2A) and was hardly detected on neurons and glia; concordantly, GPR40 mRNA expression was far greater in isolated microvessels compared with whole brain extracts (Figure 2B), despite the fact that cerebrum microvasculature only corresponds to ≈2% of total brain mass.26 We next stably transfected HEK293 cells with the hGPR40 receptor (pIRESpuro-hGPR40) to assess binding properties of TAAs on GPR40. Because all TAAs are equivalently cytotoxic on endothelial cells,18 we used 14E-AA for binding, efficacy, and mode of action. Specific binding of [14C]14E-AA was detected on pIRESpuro-hGPR40-expressing cells but hardly found in control cells transfected with the plasmid devoid of GPR40 gene; as expected, 14E-AA (as well as cis-AA) displaced bound [14C]14E-AA with an IC50 of ≈1 μmol/L (Figure 2C). Coherent with these observations, 14E-AA (5 μmol/L) specifically accrued intracellular calcium transients, extracellular signal–regulated kinase 1/2 phosphorylation, and the antiangiogenic (endothelial) proapoptotic factor thrombospondin-1 (TSP-1) in GPR40-containing cells, but not in GPR40-devoid cells (Figure 2D and 2E); the latter are consistent with extracellular signal–regulated kinase 1/2 and TSP-1–dependent TAA-induced endothelial cell death.18 Collectively, these data suggest that TAA bind and activate GPR40.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Neonatal HI Induces a Nitrative Stress-Dependent Brain Infarct Associated With Diffuse Vascular Degeneration**

Twenty-four hours after exposure of P7 rat pups to HI insult using the Rice-Vannucci model (see details in Methods in the online-only Data Supplement), we observed an infarct on the brain hemisphere (mostly cortical) ipsilateral to the carotid ligation, as well as a diffuse 40% decrease in vascular density on the same side in peri-infarct regions (Figure IA–IC). These changes were associated with a rapid (within 18 hours of insult) increase in protein and lipid nitration in ipsilateral brain parenchyma as attested by a rise in 3-nitrotyrosine adducts primarily localized on brain microvasculature (Figure 1D) and a concomitant increase in TAA (Figure 1E) to values corresponding to 2 to 5 μmol/L24; this increase in TAA was rapidly cleared as previously reported.7 As illustrated, sham, hypoxic alone, or ischemic alone control brain sections did not reveal vascular degeneration, infarct, or increases in nitration products. Inhibition of nitric oxide synthase (NOS) with intracerebroventricular L-NAME–interfered with normal retinal vascular development (detected at P8), as attested by decreased vascular area and density; the effects of 14E-AA were abrogated by GPR40 knockdown (Figure IIA–IID in the online-only Data Supplement), as we previously used for other targets.27,28 14E-AA injected intravitreally at P5 interfered with normal retinal vascular development (detected at P8), as attested by decreased vascular area and density; the effects of 14E-AA were abrogated by GPR40 knockdown (Figure IIA–IID in the online-only Data Supplement). Hence TAA-induced neuromicrovascular degeneration is GPR40 dependent.

To ascertain that TAA-induced GPR40-dependent microvascular degeneration can occur throughout the extended central nervous system, including the retina, we tested this premise in retinas knocked down or not of GPR40 for other targets.27,28 14E-AA interfered with microvascular sprouting of explants (in Matrigel) of aorta from wild-type (WT) mice, but not from congener GPR40 knockout (KO) mice (Figure 3B and 3C).

To further confirm the role of GPR40 in TAA-induced brain microvascular degeneration, we conducted in vivo experiments by injecting intracerebroventricularly 14E-AA (estimated final brain concentration ≈5 μmol/L) in mice. Twenty-four hours after injection of 14E-AA, we observed a significant decrease in periventricular cerebral microvascular density in WT, but not in GPR40 KO mice (Figure 3D and 3E).

**TAA As Bind and Activate the GPR40 Receptor**

To elucidate the specific role of TAA in HI injury in the newborn, we proceeded to identify the primary target of TAA. Because GPR40 receptor is a putative receptor for long chain FAs19,20 and cis-AA and TAAs share very high structural homology,16,25 we determined whether TAA can bind and activate GPR40. GPR40 specifically localized on cerebral microvessels (Figure 2A) and was hardly detected
by binding to its transmembrane receptor, CD36\textsuperscript{29,30}; concordantly, we hereby show that 14\textit{E}-AA induces TSP-1 in HEK293 cells, but this requires expression of GPR40 (Figure 2E). To ascertain that TSP-1 was involved in 14\textit{E}-AA–induced cerebral microvascular degeneration, we tested its effects in brain explants. 14\textit{E}-AA–induced cerebral microvascular degeneration was abrogated with an antibody that targets the binding site of TSP-1 on the CD36 receptor (Figure 4C and 4D), whereas, an antibody that targets the ox-LDL site of CD36 was ineffective. Because TAA primarily and specifically binds to GPR40 (Figure 2B and 2C), we proceeded to show its role in TAA-induced capillary drop out using GPR40 KO mice. Intracerebroventricularly injected 14\textit{E}-AA–elicited TSP-1 expression (mostly localized on microvasculature [Figure 4A]) and microvascular toxicity observed in vivo in WT mice were not detected in GPR40 KO mice (Figure 4B). These data, along with our previous report showing markedly reduced vasoobliteration in an ischemic retinopathy model in CD36 null mice,\textsuperscript{18} suggest that TAAs induce neurovascular endothelial cell death via activation of GPR40 and the subsequent generation of TSP-1 (acting via its CD36 receptor).

**HI-Induced Brain Damage Involves TAA Production and GPR40-Dependent Mechanisms**

Because neonatal HI elicits a nitrative stress-dependent increase of TAA levels (Figure 1D and 1E), which
subsequently induces endothelial cell death via activation of the GPR40 receptor (Figure 3A–3E), we determined the specific contribution of GPR40 in the cerebrovascular degeneration and infarct size triggered by HI. In contrast to the ligand TAA, expression of GPR40 was not affected by HI (Figure IV in the online-only Data Supplement). As illustrated in Figure 5, neonatal HI insult induced an ipsilateral infarct associated with decreased brain vascular density on the ipsilateral side of peri-infarct regions of WT mice. In contrast, in GPR40 KO mice exposed to the same insult, brain infarct volume was markedly reduced and microvascular density was preserved (Figure 5A–5D). Likewise, in an established ischemic retinopathy model associated with microvascular degeneration (induced by exposure of rodent pups to hypoxia) and high TAA levels, GPR40 knockdown (by injecting intravitreally a lentivirus encoded with shRNA-GPR40) prevented microvascular obliteration (Figure IIIA and IIIB in the online-only Data Supplement).

**Discussion**

Nitro-oxidative stress has been amply shown to be involved in the pathogenesis of HI brain injury of the neonate (and adult). Yet, antioxidants to date have not been successful therapeutic candidates for several reasons, which include the following: (1) compartmentalization of oxidation/nitration, (2) propensity of agent to scavenge preferentially reactive oxygen versus...
nitrogen species, (3) accessibility of antioxidants to appropriate site,31 (4) possible benefits of avoiding excess antioxidants to maintain a balanced redox potential,32,33 and (5) importantly, the induced toxic effects of stable products of oxidation/nitration (such as isoprostanes, isofurans, TAA [which are themselves not reactive]) after the oxidant stress subsides,25,34 thus acting as mediators of reactive oxygen and nitrogen species in tissue injury. Accordingly, identification of the primary site of action of stable products of oxidation/nitration would be of interest physiologically and therapeutically. Because HI brain (and retinal) injury of the newborn is significantly contributed to by nitrative stress,5–8 we proceeded to determine the primary site of action of stable products of nitro-oxidative stress, specifically TAA, because to date no specific receptors have been identified for nonenzymatic products derived from arachidonic acid. We found that TAA interact with GPR40, which in turn mediates its cytotoxic effects on neural microvascular endothelium by inducing the endothelial proapoptotic factor TSP-1; correspondingly, GPR40 participates significantly to HI brain (and retinal) injury and represents the first receptor identified to be involved in cytotoxic effects of nonenzymatically generated nitro-oxidative stress metabolites of arachidonic acid.

Nitro-oxidative stress has marked effects on lipids.35,36 These nonenzymatic reactions convert cis-AA–derived into different classes of nitro-peroxidation products, including isoprostanes, isofurans, and TAA, may also occur.17,25,37 However, these products are not only markers but also mediators of cytotoxicity induced by oxidant stress15,38,39; this claim applies to oxidative and nitrative products. As observed herein (Figure 1), HI encephalopathies and retinopathies exhibit increases in the nitro-peroxidation products, TAA, and corresponding inhibition of NOS, essential to generate TAA,16,18 prevents their concentrations from increasing and limit the vascular degeneration and the neural injury.1–8 It is noteworthy that iNOS expression does not change in neonatal HI injury,40 and although nNOS activity does,41,42 it is the perivascular eNOS that is involved in free radical production in neonatal HI43 and ensued nitration specifically localized initially on microvasculature (Figure 1D). Additional support of this inference is that eNOS KO mice do not express augmented levels of TAAs when subjected to experimental conditions susceptible to induce nitrative stress and ensued TAAs biosynthesis.18 This suggests that eNOS is the main isoform involved in TAA production in neonatal HI brain injury.

A major finding of this study is the role of GPR40, which interacts with TAA and mediates its cytotoxic effects on endothelial cells, thus explaining its involvement in HI brain (and retinal) injury (Figure 5 and Figure II in the online-only Data Supplement). We focused on GPR40 because it is a long chain ω-6 FA receptor, which interacts with lipids, such as linoleic and arachidonic acids, whereas the related GPR120 receptor interacts mostly with ω-3 FAs.21 We confirmed our presumption that TAA interact with GPR40 and the latter mediates its cytotoxic effects on neural microvasculature. GPR40 has been particularly studied for its ability to upregulate the glucose-induced insulin secretion on FA stimulation44; reported GPR40-coupled calcium mobilization and kinase

Figure 3. Transarachidonic acids induce endothelial cell death via the GPR40 receptor. A, Effects of 14E-AA, cis-arachidonic acid, and cis-linoleic acid (10−6 mol/L) on human brain microvascular endothelial cells 72 hours after transfection or not with a scrambled negative control siRNA or a siRNA targeting GPR40. B, Representative microvascular sprouting from Matrigel-embedded aortic rings harvested from wild-type (WT) and GPR40−/− knockout (KO) mice exposed to control solution or 14E-AA (5×10−6 mol/L). Scale bar=500 μm. C, Quantification of aortic ring vessel outgrowth as indicated in B (n=3–4). D, Representative brain sections illustrating the effects of intracerebroventricular injections of artificial cerebrospinal fluid (aCSF) and 14E-AA (5×10−6 mol/L) on microvascular density visualized with lectin (green) in WT and GPR40 KO mice. Scale bar=100 μm. E, Quantification of microvascular density as indicated in D (n=3).
phosphorylation^{20} partly coincide with our observations. GPR40 activated by long chain cis-FA seems to be a validated target for type 2 diabetes mellitus by enhancing glycemic control^{20,45}; yet, harmful effects mediated by GPR40 activation are still debated.^{46,47} Along these lines, activation of GPR40 by thiazolidinediones^{48} but, generally, not by long chain cis-FA, is cytotoxic^{49}; in fact, cytoprotective effects of cis-AAs is described.^{50,51} On the contrary, TAA have been consistently found to be toxic,^{16–18,25} and we now found that their effects are mediated via GPR40 (Figures 2–4). This apparent discrepancy between effects of different FAs is most likely attributable to activation of distinct allosteric sites on the receptor by different molecular structures.^{52}

The microvasculature of newborn neural tissue is particularly susceptible to nitro-peroxidant stress as observed in ischemic encephalopathies and retinopathies.^{5–8,53} In fact, injury to the microvasculature precedes that to the tissue parenchyma.^{5–7} Preservation of the microvasculature salvages (part of) the penumbral region and accordingly diminishes the size of the infarct. Our findings are consistent with these claims because inhibition of NOS which diminished formation of nitrative stress product, TAA, as well as silencing of GPR40, tended to preserve (peri-infarct) microvascular density and decreased infarct size (Figures 1 and 5).

Altogether, our findings indicate that TAAAs interact with the GPR40 receptor and mediate the microvascular degeneration induced by nitrative stress resulting from HI neural insults, providing strong evidence for an unprecedented link between GPR40 and neuromicrovascular injury in immature subjects. Accordingly, GPR40 emerges as the first receptor conveying actions of nonenzymatically generated nitro-oxidative products, namely TAA in the present case, which are importantly involved in ischemic encephalopathies (and retinopathies).

Acknowledgments
We acknowledge Dr M. Prentki (Center de Recherche du CHUM) and Drs D. Lin and H. Baribault (Amgen Inc) for providing us with the human GPR40 plasmid and the GPR40 KO breeders, respectively. We also thank G. Fergusson and M. Ethier for their invaluable technical skills and help.

Sources of Funding
This study was supported in part by grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation.
None.

Disclosures

References


GPR40 in Neurovascular Degeneration

Honoré et al


Significance

GPR40 is the first identified G protein–coupled receptor conveying actions of nonenzymatically generated nitro-oxidative products, specifically transarachidonic acids, and is involved in (neonatal) hypoxic-ischemic encephalopathy. We demonstrated that transarachidonic acids interact with GPR40 and mediate the microvascular degeneration induced by nitrative stress resulting from hypoxic-ischemic insults, providing evidence for an unprecedented link between GPR40 and neuromicrovascular injury in immature subjects. Accordingly, GPR40 emerges as the first receptor conveying actions of nonenzymatically generated nitro-oxidative products, namely transarachidonic acids in the present case, which are importantly involved in ischemic encephalopathies.
Fatty Acid Receptor Gpr40 Mediates Neuromicrovascular Degeneration Induced by Transarachidonic Acids in Rodents

Jean-Claude Honoré, Amna Kooli, David Hamel, Thierry Alquier, José-Carlos Rivera, Christiane Quiniou, Xin Hou, Elsa Kermorvant-Duchemin, Pierre Hardy, Vincent Poitout and Sylvain Chemtob

Arterioscler Thromb Vasc Biol. 2013;33:954-961; originally published online March 21, 2013; doi: 10.1161/ATVBAHA.112.300943

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/33/5/954

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/03/21/ATVBAHA.112.300943.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
THE FATTY ACID RECEPTOR GPR40 MEDIATES THE NEUROMICROVASCULAR DEGENERATION INDUCED BY TRANS-ARACHIDONIC ACIDS IN RODENTS

Supplementary Material

- Supplementary Figures

- Supplementary Figures Legends
Supplemental Figure I

A.

![Bar chart showing GPR40/β-Actin mRNA expression relative to Control.](chart_A)

B.

![Bar chart showing mRNA expression corrected to β-Actin.](chart_B)
Supplemental Figure II

A.  

14E-AA  

LV-Sh-GPR40  

+14E-AA

B.  

Vascular area (% saline)

Control  

LV-Sh-GPR40

Saline  

14E-AA

C.  

Control  

LV-Sh-GPR40

Saline  

14E-AA

D.  

Microvessel density (% saline)

Control  

LV-Sh-GPR40

Saline  

14E-AA

*
Supplemental Figure III

A.

21% O₂     80% O₂

Control    LV-Sh-GPR40

B.

Microvessel density (as normalised)

21% O₂    Control    LV-ShGPR40

80% O₂
Supplemental Figure IV

A.
**Supplementary Figures Legends**

**Supplemental Figure I.**

Downregulation of GPR40 expression using iRNA. A) GPR40 mRNA expression in human brain microvascular endothelial cells after knockdown using a siRNA targeting GPR40 or a siRNA negative control (Ambion). n = 3 from separate experiments. Values in histograms are mean ± s.e.m. *P≤0.05 compared to the untreated cells. B) Retinal GPR40 and GPR120 mRNA expressions following intra-ocular injections (P3 and P5) of a lentiviral-shRNA targeting GPR40 or a control lentiviral-shRNA. Experiment performed on P8 rat pups; n = 4 retinas. Values in histograms are mean ± s.e.m. *P≤0.05 compared to their respective control.

**Supplemental Figure II.**

Effects of GPR40 knockdown on TAA-induced retinal microvascular degeneration. (A) Representative pictures of flat-mount retinas from rat pups intravitreally injected (P3 and P5) with a lentiviral-shRNA targeting GPR40 or a control lentiviral-shRNA, and subsequently injected (P5 and P7) with 14E-AA (5x10^-6 M). Experiments performed on P8 to assess the retinal vascular area. (B) Quantification of the retinal vascular area as indicated in (A). (n=8-14). Scale bar = 100 µm. (C) Representative pictures of the retinal vascular density obtained from rat pups treated as indicated in A. Experiments performed on P8. (D) Quantification of the microvascular density as indicated in (C). (n=8-14). Scale bar = 100 µm. Values in histograms are mean ± s.e.m. *P≤0.05 compared to their respective control.

**Supplemental Figure III.**

Effects of GPR40 knockdown on oxygen-induced retinal microvascular degeneration. (A) Representative pictures of flat-mount retinas from rat pups intravitreally injected (P3 and
P5) with a lentiviral-shRNA targetting GPR40 or a control lentiviral-shRNA, and subsequently exposed from P5 to P8 to hyperoxic (80% O₂) conditions. (B) Quantification of the microvascular density as indicated in (A). (n=7-12). Scale bar = 100 µm. Values in histograms are mean ± s.e.m. *P≤0.05 compared to normoxic animals. §P≤0.05 compared to control-hyperoxic animals.

**Supplemental Figure IV.**

GPR40 expression following HI. A) GPR40 mRNA expression in mice cerebral cortex following HI. (n = 3) Values in histograms are mean ± s.e.m.
**Detailed Methods**

**Animals**

Postnatal day 7 (P7) Sprague-Dawley rat pups (Charles River) were used following approval of the research protocol by the Animal Care Committee of the CHU Ste-Justine, in accordance with guidelines of the Canadian Council on Animal Care. Female gpr40\(^{-/-}\) (KO) mice were generated as described \(^1\) and backcrossed to the C57Bl/6 strain for more than 7 generations at Amgen, Inc. (San Francisco, CA). All procedures using GPR40 KO mice were approved by the Institutional Animal Care Committee at the Centre Hospitalier de l'Université de Montréal.

**Induction of cerebral hypoxia-ischemia**

P7 rat pups or gpr40\(^{+/+}\) (wild-type: WT) and gpr40\(^{-/-}\) C57Bl/6 young adult mice (6-8 weeks of age) were anesthetized with isoflurane (2%) and subjected to unilateral carotid ligation as previously described \(^2,3\). Briefly, the left common carotid artery was ligated with surgical silk (5-0), allowed to recover for 1-2 hours, and placed in a hypoxic chamber (8% O\(_2\), balanced with nitrogen; Oxycycler, Biospherix) on a heated pad, for 90 minutes. Animals were then allowed to recover for 3h, 18h or 24 hours. Control animals were either subjected to hypoxia alone, carotid ligation alone or sham.

**Intracerebroventricular infusions and intracerebroventricular injections**

For intracerebroventricular infusions, lateral cerebral ventricles of P7 rat pups were infused with Alzet micro-osmotic pumps (Alzet) as described previously.\(^3\) Briefly, animals were anesthetized with isoflurane (2%) and osmotic pumps (0.5 µL/h infusion rate) were implanted subcutaneously (stereotaxic coordinates: PA-1.0 mm, lateral-1.0 mm from bregma and ventral-2.0 mm relative to dura) in the nuchal region. Pups were randomly selected to receive artificial cerebrospinal fluid (aCSF, vehicle) or L-NAME (2 mg/kg). For intracerebroventricular injections, a 10 µL Hamilton syringe was used. P7 rat pups or young WT and KO adult mice were injected with 5 µL of vehicle or TAA (5x10\(^{-6}\) M) over 30 seconds. Stereotaxic coordinates for young adult mice were PA-0.5 mm, lateral-1.0 mm from bregma and ventral-2.0 mm relative to dura. After 24 hours, animals were sacrificed and brains harvested for 2,3,5-Triphenyl-tetrazolium chloride (TTC; Sigma) staining or immunohistochemistry.

**Brain explants**

Brain cortex explants from rat pups (postnatal days 3 to 6) were cultured in vitro based on modifications from retinal explant protocols \(^4,5\). Brains were sectioned in ice-cold culture medium using a vibratome. Sections obtained (150 µm thick) were delicately placed in six-well dishes on top of a free-floating membrane (Nuclepore polycarbonate Track Etch, pore size 0.03 µm; Whatman). Brain explants were cultured without FBS for 3 days in Endothelial Basal Medium-2 (Clonetics) containing vehicle (control) or 14E-AA (5x10\(^{-6}\) M). Pre-treatment was performed with vehicle, an anti-CD36 mAb against the TSP-1 binding site (200 µg/mL overnight, clone FA6-152 IgG\(_1\) mouse; Beckman Coulter) or an anti-CD36 mAb against the ox-LDL binding site (100 µg/mL overnight, clone JC63.1 IgA mouse; Cayman Chemical). Ex vivo monitoring of vascular degeneration was
visualized by live-staining of endothelium using the FITC-conjugated lectin *Griffonia simplicifolia* (1:100, Sigma-Aldrich). Control explants did not show signs of vascular degeneration for up to 5 days of culture.

**Evaluation of brain infarct size**
Animals were anesthetized, perfused through the left ventricle with 4% TTC, and wrapped in an aluminium foil placed on a heated pad (37°C) for 30 minutes. Brain was then incubated in 10% formalin for 48 hours and cut into 2 mm slices. The infarct volume (mm$^3$) was calculated by measuring the infarcted area relative to the slice thickness.

**Immunohistochemistry**
Brain cryosections (10 μm) were labeled overnight at 4°C with the TRITC- or FITC-conjugated endothelial cell marker lectin *Griffonia simplicifolia* (1:100, Sigma-Aldrich), alone or in combination with a monoclonal antibody against thrombospondin-1 (TSP-1; 1:100; Clone A-6, Calbiochem) or a polyclonal antibody against 3-nitrotyrosine (3-NT; 1:200; Upstate/Millipore) or against GPR40 (1:100; Novus Biologicals), as previously described. Alexa-488-conjugated secondary antibody (1:200, Molecular Probes/Invitrogen) was applied for 60 minutes. Labeled sections were examined under an epifluorescent microscope (Eclipse E800, Nikon) equipped with a digital image analysis system (ACT-1 software, Nikon).

**Measurement of cerebral cortex vascular density**
Vascular density was quantified using the ImageJ 1.41 software (NIH, Bethesda) from pictures of brain sections labelled with the conjugated lectin. Vascular density in the cortical area was expressed as percentage of the cortical area. This technical approach to measure vascular density has already been reported in the retina and the brain.

**Synthesis of trans-arachidonic acids**
The mono-trans isomer of arachidonic acids, 14E-AA, was synthesized from the corresponding epoxide as previously described. This isomer was 100% pure and did not contain cis-arachidonic acid. Its purity and structure was confirmed by mass spectrometry, NMR, IR, HPLC, and TLC. TAA are stable as sodium salts in buffered solution. TAA remain stable when stored refrigerated (-80°C) in ethanol: no degradation or peroxidation products detected by mass spectrometry; the use of aliquots frozen for variable durations gives highly reproducible biological effects.

**Measurement of trans-arachidonic acids**
Lipids were extracted from brain cortex, separated by high pressure liquid chromatography (HPLC), and analyzed by isotopic dilution followed by gas chromatography/mass spectrometry (GC/MS) techniques as previously described. Since TAA seem to elicit comparable effects on neural vasculature, 14E-AA was measured as an indication of TAA levels.

**Aortic ring explants**
The microvascular sprouting assay was performed as reported using thoracic aortas from 6 to 8-week-old *gpr40*+/+ and *gpr40*−/− mice. Aortic rings were cultured at 37°C for
5 days in Endothelial Growth Medium-2 (Clonetics). The culture medium was changed on day 3 and the test compounds added: vehicle or 14E-AA (5x10^{-6} M). Photomicrographs of individual explants were taken before and after completion of the treatment (at day 5). The angiogenic response was determined by measuring the area of neovessel formation on computer (Image Pro Plus software; Media Cybernetics, Inc.).

**Cerebral microvessels isolation and RNA extraction**
Cerebral microvessels were prepared as previously described. Briefly, rat brains were gently homogenized with a Wheaton pestle in MEM media containing 6.3 mM HEPES and 2.5% dextran. Homogenate was then filtered through a 200 µm followed by a second filtration through a 100µm nylon mesh; the resulting filtrate was mixed with an equal volume of 40% dextran before centrifugation at 7000g for 15 minutes (4°C). The resulting pellet was washed twice with PBS and resuspended with TRizol reagent for RNA extraction, according to manufacturer’s instructions.

**Cell culture and transfection**
HEK-293E cells were stably transfected with 2 µg of either pIRES-puro-GPR40 expressing the human GPR40 receptor (provided by Marc Prentki, Montreal Diabetes Research Center, Montreal, Canada) or a control plasmid pIRES-puro using home-made prepared PEI transfection reagent (Polyethylenimine; Polysciences). Human brain microvascular endothelial cells (HBMEC) were purchased from Science Cell and cultured in ECM media containing FBS and endothelial growth supplements. Small interfering RNA experiments were performed using predesigned ready to transfec siRNA sequences targeting GPR40 or a scrambled negative control (Ambion). Cells transfected with the Lipofectamine 2000 reagent (Invitrogen) and incubated with siRNAs for 48h before performing cell viability assays. Endothelial cells of ≤5 passages were used.

**Calcium mobilization assay**
Intracellular Ca^{2+} ([Ca^{2+}]i) levels were measured using the fluorescent indicator fura 2-AM as reported by our group.

**Radioligand binding assay**
For ligand binding assay, 100,000 cells were seeded in 24-well dishes, coated with 0.1 % poly-D-lysine. After 24 hours, specific binding of [14C]14E-AA (230 nmol/L) was determined in the presence of cold 14E-AA (40 μM). This radioligand assay was carried out on attached cells for 90 minutes on ice in binding buffer (10 mmol/L PBS/0.05 % BSA). The amount of bound radioactivity was determined after washing twice with cold binding buffer and lysing cells in 0.1N NaOH/0.1 % Triton X-100.

**Cell viability assay**
MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were performed to assess cell viability as we previously described, after quiescent cells were starved and treated with either vehicle or 14E-AA for 24 hours.
Western blotting
Standard SDS-PAGE techniques were followed as previously described \(^7\). Primary antibodies were used according to the following conditions: p-erk1/2 (1:1000 dilution, Cell Signaling) and TSP-1 (1:200, Calbiochem). Equal protein loading was insured by probing with 1:200 total erk antibody (Santa Cruz) and 1:50,000 \(\beta\)-actin antibody (Novus Biologicals), correspondingly. Densitometry was measured in pixel intensity by Image-Pro Plus.

Quantitative Real time PCR
Primer sequences have been designed using the DNAMAN software (Lynnon Inc., Pointe-Claire, QC, Canada). The following primers were used for qPCR experiments: Rat (r) primers had the following forward (F) and reverse (R) sequences: rGPR40 (F) 5’-CTATATGGGCCCTTGTC C TTT-3’ and (R) 5’-AATCGAGAAACTGAGTCGGG-3’ while beta-actin primers were selected according to Sullivan et al. \(^17\). Primers targeting mouse GPR40 are: (F) 5’-CTTCGGGTACCAAGCCATCC-3’ and (R) 5’-ACTGGTACTGGTGGTGCCAGGC-3’. Specificity of the primers designed using the DNAMAN software has been evaluated according to the nucleotide BLASTN program as well as the size of the amplified products on 2% agarose gels.

Chemicals and Materials
Trans-arachidonic acids and \([^{14}\text{C}]1\text{E}-\text{AA}\) were a kind gift from Drs. Emile Falek and Michael Balazy. Linoleic acid, cis-arachidonic acid, arachidonic acid-5,6,8,9,11,12,14,15-\(^3\text{H}(\text{N})\), lectin FITC-coupled and TRITC-coupled were purchased from Sigma Aldrich Inc.

Statistical analysis
Data were analyzed by two-way ANOVA followed by Bonferroni post test. Values are presented as means ± S.E.M. Statistical significance was set at \(p<0.05\).
References


