Formyl-Peptide Receptor 2/3/Lipoxin A₄ Receptor Regulates Neutrophil-Platelet Aggregation and Attenuates Cerebral Inflammation
Impact for Therapy in Cardiovascular Disease

Shantel A. Vital, MS; Felix Becker, MD; Paul M. Holloway, PhD; Janice Russell; Mauro Perretti, PhD; D. Neil Granger, PhD; Felicity N.E. Gavins, PhD

Background—Platelet activation at sites of vascular injury is essential for hemostasis, but it is also a major pathomechanism underlying ischemic injury. Because anti-inflammatory therapies limit thrombosis and antithrombotic therapies reduce vascular inflammation, we tested the therapeutic potential of 2 proresolving endogenous mediators, annexin A1 N-terminal derived peptide (AnnxA1Ac2–26) and aspirin-triggered lipoxin A₄ (15-epi-lipoxin A₄), on the cerebral microcirculation after ischemia/reperfusion injury. Furthermore, we tested whether the lipoxin A₄ receptor formyl-peptide receptor 2/3 (Fpr2/3; ortholog to human FPR2/lipoxin A₄ receptor) evoked neuroprotective functions after cerebral ischemia/reperfusion injury.

Methods and Results—Using intravital microscopy, we found that cerebral ischemia/reperfusion injury was accompanied by neutrophil and platelet activation and neutrophil-platelet aggregate formation within cerebral microvessels. Moreover, aspirin-triggered lipoxin A₄ activation of neutrophil Fpr2/3 regulated neutrophil-platelet aggregate formation in the brain and inhibited the reactivity of the cerebral microvasculature. The same results were obtained with AnnxA1Ac2–26 administration. Blocking Fpr2/lipoxin A₄ receptor with the antagonist Boc2 reversed this effect, and treatments were ineffective in Fpr2/3 knockout mice, which displayed an exacerbated disease severity, evidenced by increased infarct area, blood-brain barrier dysfunction, increased neurological score, and elevated levels of cytokines. Furthermore, aspirin treatment significantly reduced cerebral leukocyte recruitment and increased endogenous levels of aspirin-triggered lipoxin A₄ effects again mediated by Fpr2/3.

Conclusion—Fpr2/lipoxin A₄ receptor is a therapeutic target for initiating endogenous proresolving, anti-inflammatory pathways after cerebral ischemia/reperfusion injury. (Circulation. 2016;133:2169-2179. DOI: 10.1161/CIRCULATIONAHA.115.020633.)

Key Words: inflammation ■ ischemia reperfusion injury ■ stroke, ischemic

Inflammation plays a crucial role in the pathophysiological cascade of ischemic stroke and related forms of brain injury. Although the exact mechanisms responsible for post-ischemic cerebral damage are not fully understood, increasing evidence suggests the inflammatory state after ischemia/reperfusion (I/R) as a crucial contributing factor to these pathogenic processes. It has been demonstrated that post-I/R inflammation results in endothelial cell activation and consequent recruitment and activation of leukocytes and platelets, leading to further cerebral microvascular dysfunction and subsequent tissue injury. Because the brain is highly susceptible to I/R injury, prevention of cerebral ischemic events has become a major part of modern health care. However, neuronal cells have a limited capacity for restoration after cerebral I/R; therefore, there is an unmet clinical need to explore novel therapeutic avenues to better resolve the aggressive inflammatory state after cerebral ischemic insults to limit the irrecoverable neuronal damage.

Editorial, see p 2128
Clinical Perspective on p 2179

It is now appreciated that inflammatory mechanisms do not disperse spontaneously but rather that resolution of inflammation involves a tightly orchestrated series of events, engaging specific endogenous mediators and protective pathways. Among these, annexin A1 (AnnxA1) and its N-terminal peptide AnnxA1Ac2–26 (Ac-AMVSEFLQKWFIENEEQEVQTVK), the eicosanoid-derived lipoxin A₄ (LXA₄), and 15(R)-epi-lipoxin A₄ (referred to as aspirin-triggered lipoxin [ATL] and produced after aspirin acetylation of inducible cyclooxygenase-2) have been shown...
to play an active role in returning local sites of inflammation to homeostasis. These proresolving mediators act at different phases of the inflammatory response and are involved in blockade of leukocyte recruitment, inhibition of cytokine release, promotion of apoptosis, stimulation of phagocytosis, and decreasing vascular permeability. Effector cells of resolution ensure that these responses remain localized within the inflamed tissue and are extinguished within an adequate timeline. Of relevance, both AnxA1/AnxA1Ac2–26 and LXA4/ATL mediate their effects through Fpr2/3, also called lipoxin A4 receptor (ALX). This receptor is expressed on a variety of cell types such as endothelial cells and myeloid lineages, for example, neutrophils, which are involved in the inflammation observed in early stages of reperfusion after ischemic insults.

Several lines of evidence, produced by our group and others, indicate protective effects of these proresolving mediators in a number of post-I/R events, including in the mesentery, heart, lung, and kidney. We previously shown that AnxA1/AnxA1Ac2–26 and LXA4/ATL mediate their effects through Fpr2/3 in a global murine stroke model (a model that mimics the carotid arteries and respiratory or cardiac arrest). In a focal I/R model (the middle cerebral artery occlusion + reperfusion [MCAo/R] model), we reported that exogenous administration of AnxA1 markedly attenuated I/R injury by acting on a member of the FPR family, which at the time we speculated to be Fpr2/3 mice. We present novel evidence that specifically neutrophils and platelets, which are involved in the inflammation observed in early stages of reperfusion after ischemic insults.

Here, we demonstrate a novel role of AnxA1Ac2–26 and ATL in regulating leukocyte-platelet responses in the cerebral microvasculature after I/R as attained with pharmacological blockade, quantifying endogenous mediator levels, and using Fpr2/3−/− mice. We present novel evidence that specifically neutrophil Fpr2/3 regulates cerebral neutrophil-platelet aggregation (NPA) by rapid generation of ATL in an endogenous biosynthetic circuit to resolve inflammation and to restore homeostasis. In summary, Fpr2/ALX is a therapeutic target for initiating endogenous proresolving, anti-inflammatory pathways after cerebral I/R injury.

**Methods**

A detailed Methods section is provided in the online-only Data Supplement.

**Animals**

Animal experiments complied with ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines and followed the European Union Directive (2010/63/EU) or Louisiana State University Health Sciences Center Shreveport Institutional Animal Care and Use Committee. Wild-type (WT) C57BL/6 mice or Fpr2/3−/− mice were used.

**Receptor Agonists and Drug Treatment**

Vehicle (saline or ethanol plus saline for ATL), AnxA1 mimetic peptide Ac2-26 (AnxA1Ac2–26; Ac-AMVSEFLQKAWFIEEQQEYQTVK, Cambridge Research Biochemicals, Cleveland, UK) 100 µg per mouse. Boc2 (N-tetra-butoxycarbonyl-l-Phe-o-Leu-l-Phe-o-Leu-l-Phe, MPBiomedicals, Cambridge, UK) 10 µg per mouse, and ATL (stable epimer of LXA4) 4.0 µg per mouse were administered (100 µL) intravenously at the start of cerebral reperfusion. In a separate set of experiments, mice were intraperitoneally treated with aspirin (ASA; 150 mg/kg, Sigma-Aldrich, Dorset, UK) 60 minutes before I/R.

**Middle Cerebral artery Occlusion + Reperfusion (MCAo/R) Model**

MCAo was performed for 60 minutes followed by 4 or 24 hours of reperfusion according to standard operating procedure in our laboratory.

**Blood Sampling and Cell Counts**

Blood samples were taken before and after MCAo for measuring leukocytes (3% citric acid and 10% crystal violet) for platelet (1% buffered ammonium oxalate) counts with a hemocytometer.

**Platelet and Leukocyte Labeling**

Platelets were isolated from donor mice, labeled with carboxyfluorescein succinimidyl ester (CFSE 90 µmol/L, 10 minutes, Sigma-Aldrich) and injected into recipient mice, followed by 0.02% rhodamine 6G (Sigma-Aldrich) to label circulating leukocytes.

**Cerebral Intravitral Fluorescence Microscopy**

Intravitral fluorescence microscopy (IVM) was performed with a Zeiss Axioskop microscope (Zeiss, New York, NY) (see the online-only Data Supplement).

**Infarct Volume**

After 24 hours of reperfusion, brains were removed and stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma Aldrich). Sections were photographed, and the digitized images of each brain section (and the infarcted area) were quantified with NIH 1.57 Image Software.

**Neurological Score**

A five-point neurological deficit score (NS) was used: 0=no deficit, 1=failure to extend the right paw, 2=circling to the right, 3=falling to the right, and 4=unable to walk spontaneously.

**Blood-Brain Barrier Dysfunction**

Blood-brain barrier (BBB) permeability was assessed with Evans blue extravasation. BBB permeability was normalized by dividing tissue Evans blue concentration (µg/g brain weight) by plasma concentration (µg/mL).

**Myeloperoxidase Activity**

Brain homogenates and myeloperoxidase standards (Sigma-Aldrich) were placed on a 96-well plate, and 200 µL o-dianisidine (Sigma-Aldrich) solution and 10 minutes, Sigma-Aldrich). Plates were placed on a 96-well plate, and 200 µL of 0.1% H2O2 (Sigma-Aldrich) were added. Absorbance was read after 5 minutes at 405 nm and expressed as units per 1 mg wet tissue.

**Confocal Microscopy**

To visualize NPAs, mice were injected with specific antibodies to label neutrophils (eFluor 660 [green]–labeled anti-mouse Ly-6G, eBioscience, San Diego, CA; 2 µg per mouse) and platelets (Dylight 649 [red]–labeled anti-mouse CD42, Dallal-Wilkinson-Lillie formula for corrected P values.

**Cytokines in Plasma and Brain Tissue**

After 24 hours of reperfusion, plasma and brain hemisphere homogenates were obtained. Levels of proinflammatory and anti-inflammatory cytokines, ATL, and thromboxane B2 were measured with standard ELISAs as described in the online-only Data Supplement.

**Statistical Analysis**

Results from IVM experiments were confirmed to follow a normal distribution with the Kolmogorov-Smirnov test of normality with Dallal-Wilkinson-Lillie formula for corrected P values. Data that
Results

Cellular Responses Are Exacerbated in the Cerebral Microcirculation of Fpr2/3−/− Mice

MCAo for 60 minutes followed by 4 or 24 hours of reperfusion induced heightened cellular trafficking within the cerebral microcirculation in both WT and Fpr2/3−/− mice compared with sham animals, with an enhanced inflammatory response in Fpr2/3−/− mice. At 4 hours, Fpr2/3−/− mice displayed a significant increase in leukocyte (but not platelet) adhesion compared with WT mice at that time point. Furthermore, at 24 hours, this inflammatory response was more pronounced, and Fpr2/3−/− demonstrated an increase in both leukocyte and platelet adhesion (P<0.0001 for both) compared with WT mice (Figure 1A and 1B). Figure 1C and 1D illustrates representative images of cerebral venules from sham, I/R WT, and Fpr2/3−/− mice at 24 hours, displaying the marked accumulation of leukocytes and platelets (Figure 1E and 1F).

Mice Lacking Fpr2/3 Have Increased Cerebral Damage After I/R

NS and infarct volume (IV) in WT and Fpr2/3−/− mice subjected to 60 minutes of MCAo with 24 hours of reperfusion or sham are presented in Figure 2A and 2B. Although after 4 hours of reperfusion differences were noted between sham and I/R, no changes were noted between the genotypes (data not shown). However, NSs assessed after 24 hours of reperfusion were significantly worse in Fpr2/3−/− than in WT mice (Figure 2A), a response that correlated with elevated IVs (Figure 2B). No significant differences among mouse genotypes were detected with respect to the hemodynamic parameters under assessment (Table I in the online-only Data Supplement).

Fpr2/3−/− Mice Have Augmented Cerebral and Systemic Inflammatory Responses After I/R

We further investigated the involvement of Fpr2/3−/− in local and systemic inflammatory responses. BBB integrity was monitored in WT and Fpr2/3−/− mice at 24 hours of reperfusion with the Evans blue extravasation method. Figure 2C shows that BBB...
Permeability was significantly elevated in the left (injured) hemisphere of WT and Fpr2/3−/− mice after I/R compared with their respective contralateral side. Sham animals had no BBB dysfunction (data not shown). Most important, compared with WT mice, Fpr2/3−/− mice showed a significantly higher Evans blue extravasation, indicating an exacerbated BBB dysfunction (Figure 2C). Furthermore, we found a marked increase in myeloperoxidase (a marker of neutrophil activation) after I/R, which again was significantly higher in Fpr2/3−/− mice (Figure 2D).

All mice undergoing MCAo/R had decreased white blood cell counts, which was reflected by a decrease in lymphocyte count. The neutrophil count was increased in MCAo/R mice and further exacerbated in MCAo/R Fpr2/3−/− mice compared with WT MCAo/R mice (Table). No differences were noted in platelet count (Table), the relative proportions of immature versus mature platelets (Figure IA and IB in the online-only Data Supplement), or the level of activation within these populations (Figure IC and ID in the online-only Data Supplement) in any groups or genotypes.

Finally, we determined serum and tissue cytokine levels. All cytokines (except interleukin [IL]-10) measured in plasma of WT mice were elevated after I/R compared with sham mice (data not shown), whereas in the brain homogenates, MCAo/R elicited discrete increases in tumor necrosis factor-α (TNFα), CCL2, and

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Lack of formyl-peptide receptor 2/3 (Fpr2/3) leads to increased tissue damage in the brain after ischemia/reperfusion (I/R). Wild-type (WT; C57BL/6) and Fpr2/3−/− mice were subjected to middle cerebral artery occlusion for 60 minutes followed by 24 hours of reperfusion. A, Neurological score was assessed and brains were removed to quantify (B) infarct volume. In a separate cohort of animals, brain homogenate samples were taken. C, Blood-brain barrier dysfunction was measured in the right and left hemispheres of mice with Evans blue (EB) dye extraction. D, Myeloperoxidase (MPO) was measured in whole-brain homogenates. "P<0.01, ""P<0.001 vs control. ##""P<0.001 vs genotype at same time point.

### Table. Cell Counts of WT and Fpr2/3−/− Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Total WBC Count, ×10⁹/L</th>
<th>Neutrophils per 1 μL blood, n</th>
<th>Monocytes per 1 μL blood, n</th>
<th>Lymphocytes per 1 μL blood, n</th>
<th>Platelets per 1 μL blood, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Sham</td>
<td>6.7±0.2</td>
<td>1697±47.0</td>
<td>875.5±55.1</td>
<td>8322±563.2</td>
<td>993.8±17.3</td>
</tr>
<tr>
<td></td>
<td>I/R</td>
<td>4.3±0.3</td>
<td>3728±538.4</td>
<td>1001±210.4</td>
<td>1508±85.93</td>
<td>980.0±35.2</td>
</tr>
<tr>
<td>Fpr2/3−/−</td>
<td>Sham</td>
<td>5.7±0.4</td>
<td>1365±250.3</td>
<td>953.0±87.7</td>
<td>7967±468.0</td>
<td>861.3±29.4</td>
</tr>
<tr>
<td></td>
<td>I/R</td>
<td>3.6±0.2</td>
<td>5467±452.2</td>
<td>1295±102</td>
<td>2133±283.6</td>
<td>855.8±34.7</td>
</tr>
</tbody>
</table>

I/R indicates 60 minutes of ischemia plus 24 hours of reperfusion; WBC, white blood cell; and WT, wild-type.

*P<0.05, **P<0.001 vs sham of the same genotype.

§P<0.05 vs WT I/R.
IL-6 compared with sham mice (data not shown). When comparing WT and Fpr2/3−/− mice subjected to MCAo for 60 minutes followed by 24 hours of reperfusion, we found that that absence of Fpr2/3−/− resulted in heightened serum levels of IL-1β and TNFα (Figure 3A and 3B) with no significant difference in CCL2, IL-10, and IL-6 levels compared with WT mice (Figure 3C–3E).

Local cytokine levels (TNFα, CCL2, and IL-6) were significantly elevated in the left (injured) hemisphere of WT and Fpr2/3−/− mice after I/R (Figure 3G, 3H, and 3J). Moreover, both TNFα and IL-1β levels were more elevated in Fpr2/3−/− mice than in WT mice. IL-10 levels were decreased in Fpr2/3−/− mice compared with WT mice. No cytokine was above control values in the contralateral hemisphere of either WT or Fpr2/3−/− mice (except IL-10), suggesting that these inflammatory mechanisms are confined to the ischemic region (ie, ipsilateral side).

**Fpr2/3−/− Mice Have Reduced Cerebral NPAs**

Once stimulated at sites of inflammation, activated platelets are prone to adhering to neutrophils, forming circulating NPAs, a mechanism that has been demonstrated in ischemic stroke.21 Because the IVM data suggested an augmented platelet activation in Fpr2/3−/− mice (Figure 1B), we further investigated the state of activation in platelets after cerebral I/R injury by measuring the thromboxane A₂ metabolite thromboxane B₂, which is a soluble marker for platelet activation. Plasma levels of stable thromboxane B₂ were increased in both WT and Fpr2/3−/− mice after I/R and further elevated in the absence of Fpr2/3 when we compared I/R groups with each other (Figure 4A). We then assessed the potential of these activated platelets to form aggregates. By using fluorescence IVM, we revealed a dynamic formation of NPAs within the brain microcirculation, showing intravascular neutrophils interacting with platelets (Figure 4B). Surprisingly, we found that Fpr2/3−/− mice subjected to MCAo for 60 minutes followed by 24 hours of reperfusion had significantly fewer NPAs than WT mice (Figure 2C). Although the IVM data had shown an increase in total adherent leukocyte counts in Fpr2/3−/− mice compared with WT mice, we found that ≈33% of these were platelet bearing in Fpr2/3−/− mice compared with

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**Figure 3.** Inflammatory responses are augmented in formyl-peptide receptor 2/3–deficient (Fpr2/3−/−) mice. Wild-type (WT; C57Bl/6) and Fpr2/3−/− mice were subjected to middle cerebral artery occlusion for 60 minutes followed by 24 hours of reperfusion, after which plasma and brain homogenate samples were taken. Cytokines were measured in plasma (A–E) and homogenates from the left (ipsilateral) and right (contralateral) sides of the brain (F–J). Data are means±SEM of 8 mice per group. IL indicates interleukin; and TNFα, tumor necrosis factor-α. **P<0.01, ***P<0.001 vs same genotype right side. ###P<0.001 vs WT left side.
≈60% platelet-bearing leukocytes in WT mice (No significant differences were measured between WT and Fpr2/3−/− mice in sham conditions).

Next, it was important to ascertain whether platelet or neutrophil Fpr2/3 was functional in these settings. Data in Figure 4D show that after I/R CSFE-labeled platelets from Fpr2/3−/− mice injected into WT animals led to numbers of NPAs similar to those quantified with WT platelets transferred into WT recipients. Thus, NPA formation in WT animals was independent of the presence of Fpr2/3 on the infused platelets. In addition, lower aggregate numbers were found when platelets from WT or Fpr2/3−/− mice were injected into Fpr2/3−/− mice subjected to I/R (Figure 4D), demonstrating that NPA formation in Fpr2/3−/− mice was also independent from the type of transferred platelets. Thus, these findings reveal that it is not the platelet Fpr2/3 that is important in NPA formation but rather a functional neutrophil Fpr2/3.

**Fpr2/3 Agonists Attenuate I/R-Induced Cerebrovascular Injury in WT Mice**

Having demonstrated an exacerbated cerebral inflammatory response in mice lacking Fpr2/3, coupled with the importance for a functional neutrophil Fpr2/3, we next tested the protective potential of targeting FPR2/ALX in stroke. Treating WT mice with either AnxA1Ac2–26 or ATL markedly attenuated the inflammatory response, as assessed by NS, IVM, and IV (Figure II in the online-only Data Supplement). No significant differences in parameters measured were observed between either vehicle (saline or saline plus ethanol), so only saline vehicle is presented. Intravenous administration of AnxA1Ac2–26 to I/R WT mice reduced the number of adherent leukocytes (303.0±23.5 cells/mm²; Figure 5A) and platelets (92.1±10.1 cells/mm²) compared with saline-injected animals (433.8±15.8 and 158.5±10.0 cells/mm², respectively). These protective effects translated to reduced IV (17.2±1.3% versus 25.3±1.2% in saline-treated mice) and NS (1.5±0.2 versus 2.9±0.2). Similar effects were produced with ATL, decreasing leukocyte adhesion by 35%, platelet adhesion by 45%, IV by 42%, and NS by 58% compared with WT mice. When either compound was coadministered with the Fpr2/3 antagonist Boc2, there was a loss of effect (Figure II in the online-only Data Supplement), whereas the antagonist was ineffective when given alone.

**Harnessing Endogenous Fpr2/3 Activation as a Therapeutic Strategy**

Finally, we assessed whether a pharmacological dose of ASA (150 mg/kg) changed ATL levels in our stroke model. Plasma
and brain homogenate ATL concentrations were measured after cerebral I/R with and without ASA treatment. ASA induced a significant increase in both plasma and brain homogenate ATL levels compared with vehicle-treated WT mice (Figure 5A and 5B). Furthermore, ASA treatment reduced leukocyte adherence and NPAs in WT mice (Figure 5C and 5D). Next, we determined whether increased levels of endogenous ATL produced by ASA treatment contributed to the cerebroprotective effects that we observed. Administration of the Fpr2/3 antagonist Boc2 alone15 had no effect on leukocyte adhesion and NPA formation. However, when administered in combination with ASA, it reversed the effects of ASA; that is, it increased leukocyte adhesion and NPA formation (Figure 5C and 5D). These pharmacological results were complemented by analysis in Fpr2/3−/− mice. Despite the fact that administration of ASA to Fpr2/3−/− and WT mice resulted in increased plasma and brain homogenate ATL concentrations (Figure 5E and 5F), ASA was unable to reduce leukocyte adherence or NPAs in Fpr2/3−/− mice (Figure 5G and 5H). Furthermore, we found that ASA was unable to reduce NS and IV in Fpr2/3−/− mice but was able to do so in the WT counterparts (data not shown).

Discussion
There are several novel key findings from this study: (1) The absence of Fpr2/3 heightens the inflammatory response after cerebral I/R; (2) agonistic targeting of Fpr2/3 is protective in stroke; (3) ATL is required for ASA protection in cerebral I/R injury; and finally, (4) functional neutrophil Fpr2/3 is needed for formation of NPAs, through which ATL exerts its effects within the cerebral microvasculature (Figure 6).

Despite the positive effects of blocking various aspects of the inflammatory cascade, which ameliorates injury in experimental stroke,20 there are currently few effective treatment options for patients affected by ischemic stroke. The second phase of inflammation is the resolution one in which active engagement of molecular and cellular circuits ensures termination.5 Thus, endogenous anti-inflammatory mediators such as AnxA1Ac2-26 and ATL could be exploited for guiding innovative drug discovery programs to develop compounds likely to be devoid of the major side effects associated with current anti-inflammatory and vasculoprotective therapies (eg, vomiting, nausea, constipation, diarrhea, ulcers, bleeding, organ [liver and kidney] failure) because their application would mimic a way that the body naturally disposes of inflammation.22 These mediators target Fpr2/3; hence, we began here by assessing whether Fpr2/3 played functional roles in cerebral I/R.

Fpr2/3 belongs to a receptor family of the hematopoietic/myeloid cell population. Within this family, FPR1 is one of the best-studied G protein–coupled receptors, dating back to the
Three functional receptors (FPR1, FPR2/ALX, and FPR3) exist in humans (and mice) with a wide tissue distribution; they interact with a number of structurally diverse, naturally occurring ligands that can promote opposing biological actions. We demonstrate here that Fpr2/3−/− mice (lacking the ortholog of FPR2/ALX) display an exacerbated cerebral inflammatory response after I/R injury. This was observed by increased adherent leukocytes and platelets, which is in keeping with the cerebral effects observed in other models (eg, systemic administration of lipopolysaccharide), along with increased IV (which is inextricably linked to levels of inflammation during cerebral reperfusion) and NS. We also found that although there was a reduced circulating lymphocyte count in MCAo/R mice (which may account for the decrease in white blood cells and confirms findings from another group), there was no difference between WT and Fpr2/3−/− mice. However, the exacerbation detected in peripheral neutrophil counts in MCAo/R Fpr2/3−/− compared with MCAo/R WT mice may account for the increased adherent neutrophils quantified in the brain microvasculature of these mice. These findings are in line with the experimental and clinical notion that supports the importance of neutrophil infiltration in ischemic stroke.

Previous focal I/R models have shown that the recruitment of neutrophils in the ischemic brain occurs within 30 minutes to a few hours and peaks in the first 3 days. Furthermore, in the clinic, neutrophil counts are increased in the first 3 days after symptom onset in stroke patients, and this is associated with larger final IVs (on computed tomography and magnetic resonance imaging) and increased stroke severity.

Because stroke is associated with platelet activation, which we monitored by the heightened platelet adhesion in the brain, we determined whether Fpr2/3 deficiency changes the number of total, immature, and mature platelets and the level of activation within these platelet populations before and after stroke. Although we noted no change in platelet count resulting from stroke or between genotypes, we investigated this further using thiazole orange, a nucleic acid intercalating dye that has previously been used to detect and quantify immature platelets (which contain more RNA than their mature counterparts) to assess the number of both mature and immature circulating platelets and the level of activation within these populations (by using the antibody [JONA] that recognizes the activated conformation of the murine glycoprotein IIb/IIIa integrin on the platelet surface). These data

Figure 6. Schematic overview of the important anti-inflammatory role that formyl-peptide receptor 2/3 (Fpr2/3) plays in abrogating ischemia/reperfusion (I/R)–induced cerebrovascular injury. I/R leads to the production of Fpr2-mediated formation of neutrophil/platelet aggregates (NPAs), with neutrophil Fpr2/3 mediating the signal. Aggregates allow the biosynthesis of lipoxin A4 (LXA4) via neutrophil 5-lipoxygenase (5-LOX) and platelet 12/15-LOX. High levels of aspirin-triggered lipoxin (ATL) are also generated during I/R. The effect of ATL on the cerebral microcirculation was absent in mice that lack the Fpr2/3, but pharmacological targeting of the Fpr2/3 (with AnxA1Ac2–26 or ATL) can resolve these inflammatory effects. The administration of aspirin (ASA) is able to produce ATL via cyclooxygenase-2 (COX2) acetylation, which in turn acts on Fpr2/3 and monitors the vascular permeability in the brain. In summary, Fpr2/3 (and we propose the human counterpart, lipoxin A4 receptor / FPR2) promotes the biosynthesis and production of lipoxins and ATL, which in turn regulates the inflammatory process and restores homeostasis in the brain.
confirmed that the relative proportions of immature versus mature platelets did not change and that the level of activation within these populations was also unchanged. These observations demonstrate that the effects of our MCAo/R on platelet activation are local, whereas levels of circulating platelets and their maturity are not affected.

The BBB is a critical guardian of communication between the periphery and the brain and was compromised to a greater degree in the ipsilateral side compared with WT mice, suggesting that Fpr2/3, either directly or indirectly, preserves BBB permeability. This concurs with previous observations in which this receptor has been shown to be involved in the maintenance of BBB structure and function by downregulating RhoA GTPase activity after binding of AnxA1 and, in so doing, enabled the organization of the interendothelial cell tight and adherens junctions.

Overexpression of proinflammatory cytokines is another important component of the neuroinflammatory response. The precise concoction of proinflammatory cytokines deployed during I/R is variable, especially when assessed in the brain and not the periphery. We found an increase in IL-6 in I/R animals, which is in keeping with clinical data (>10 pg/mL in patients) and can correlate with poor clinical outcomes. We measured higher levels of TNFα and IL-1β after cerebral I/R (heightened in the Fpr2/3−/− mice). These cytokines not only attract leukocytes into ischemic sites, promoting tissue necrosis, but also can damage the microvascular endothelium, disrupt the BBB, and accelerate the formation of cerebral edema. We also found low IL-10 levels in both plasma and brain homogenates in the Fpr2/3−/− mice. This is fitting with the clinical situation found low IL-10 levels in both plasma and brain homogenates. That being said, we have not ruled out the possibility that the generation of other Fpr2/3 agonists (especially LXA4 and its derivatives) are able to decrease myeloperoxidase levels. This could be because lipoxins are able to inhibit neutrophil migration, enhance neutrophil apoptosis, and in some cases suppress reactive oxygen species production.

Growing evidence suggests that platelet-derived microparticles amplify leukocyte activation and transmigration. Augmented plasma levels of TXB2 after I/R suggest amplified platelet activation, which was markedly increased in FPR2/3−/− mice. Previous reports of NPA formation in inflammation have shown that neutrophils bound to the endothelium secondarily capture platelets and that NPA formation and platelet activation are increased in ischemic stroke and are involved in the pathogenesis of stroke. However, interestingly, our findings demonstrated for the first time that NPAs formed in the cerebral microcirculation in animals after I/R were significantly less in Fpr2/3−/− compared with WT animals. It is known that neutrophils can use platelet-derived arachidonate to generate lipoxigenase products and that NPA interactions use bidirectional transcellular routes to contribute to LXA4 and ATL formation. Our results correlate with these findings and those of Brancaleone et al in the mesenteric microcirculation that neutrophil/platelet interactions allow the production of LXA4. Here, we also found increased levels of ATL in both plasma and brain homogenates. That being said, we have not ruled out the possibility that the generation of other Fpr2/3 agonists could be contributing to the lipid mediator release. Some examples are resolvins and humanin. Further experiments will shed light on this.

Neutrophil accumulation is fundamental to acute inflammation. In response to tissue injury, circulating NPAs form for secondary capture. Counterregulation of acute inflammatory processes by specialized proresolving mediators is essential to restore homeostasis. In peripheral organs such as the mesentery and lung, endogenous biosynthetic routes have been shown to be amplified during NPA formation to restrain these aggregates with counterregulatory circuits used to regulate the extent of the inflammation and to protect the tissue from further damage. Although this has been shown in systemic organs, the effect in the brain microcirculation was unknown. Using adoptive transfer of donor platelets into recipient mice and similar strategies, we concluded that in the cerebral microvasculature, neutrophil Fpr2/3 is important in forming aggregates with platelets. Although these findings are in line with previous findings in the systemic circulation (ie, mesentery) after I/R injury, it would appear that in a lipopolysaccharide-induced lung injury model, neither neutrophil nor platelet Fpr2/3 alone is required
for the formation of aggregates but rather that Fpr2/3 on both cell types is necessary to inhibit inflammation.\(^4\) Additionally, it cannot be ruled out that the engagement of Fpr2/3 on other cell types such as endothelial cells\(^8\) or microglia\(^2\) could play a role in these findings. However, Fpr2/3 expression levels are very low in endothelial cells\(^23\) and thus are unlikely to have an impact on the NPA response observed here.

Finally, to establish the clinical relevance of Fpr2/3 as a therapeutic target for stroke treatment and other neurovascular diseases such as multiple sclerosis\(^43\) and Alzheimer disease,\(^44\) we determined its impact in the pharmacological actions of ASA. This nonsteroidal anti-inflammatory drug has the ability to activate a number of mechanisms such as interfering with transcription factor of adenosine signaling\(^45\) and the acetylation of cyclooxygenase-2 to block prostanoid synthesis.\(^45\) Contact between platelets and neutrophils gives rise to the transcellular production of lipoxins and leukotrienes,\(^45\) and even low-dose ASA administered to healthy volunteers is capable of producing bioactive levels of ATL.\(^33\) Here, we showed that ASA administered to healthy volunteers is capable of producing ATL, which triggers proresolving responses through the engagement of Fpr2/3.

Conclusions
Our results demonstrate that an absence of Fpr2/3 leads to an aberrant inflammatory response to stroke and that compounds targeting Fpr2/3 eg, AnxA1,\(^\text{Ad}2-29\), and ATL are powerful inhibitors of these inflammatory events. Our findings shed light on an endogenous biosynthetic circuit in neutrophils in which neutrophil Fpr2/3 controls NPA formation by rapid generation of ATL, which is engaged to restrain NPA formation and acute inflammation and to restore homeostasis in the brain. Furthermore, this study shows that Fpr2/3 is an attractive therapeutic target and that ATL and other potentially proresolving lipid mediators are attractive treatment option for patients with stroke, a disease that currently lacks any effective pharmacotherapies.

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References


the novel role of AnxA1Ac2-26 and aspirin-triggered lipoxin in regulating leukocyte-platelet responses in the cerebral microcirculation during reperfusion; therefore, there is an unmet clinical need to explore novel therapeutic avenues to better resolve the aggressive inflammatory state after cerebral ischemic insults to limit irrecoverable neuronal damage. Our study shows for the first time the novel role of AnxA1Ac2-26, and aspirin-triggered lipoxin in regulating leukocyte-platelet responses in the cerebral microcirculation after ischemia/reperfusion. These results were attained by pharmacological blockade, quantification of endogenous mediator levels, and the use of formyl-peptide receptor 2/3-deficient (Fpr2/3−/−) mice. Furthermore, we demonstrate that specifically neutrophil Fpr2 regulates cerebral neutrophil-platelet aggregation by rapid generation of aspirin-triggered lipoxin in an endogenous biosynthetic circuit. This pathway helps to resolve inflammation and to restore homeostasis. This has been shown for the first time in the brain, and we feel that these data are exciting because they confirm that Fpr2/lipoxin A2 receptor is a therapeutic target for initiating endogenous proresolving, anti-inflammatory pathways after cerebral ischemia/reperfusion injury, which has clear clinical implications in helping to limit neuronal damage after ischemic insults.

CLINICAL PERSPECTIVE

The brain is highly susceptible to ischemia/reperfusion injury, so prevention of cerebral ischemic events has become a major part of modern health care. However, neuronal cells have a limited capacity for restoration after cerebral ischemia/reperfusion; therefore, there is an unmet clinical need to explore novel therapeutic avenues to better resolve the aggressive inflammatory state after cerebral ischemic insults to limit irrecoverable neuronal damage. Our study shows for the first time the novel role of AnxA1Ac2-26, and aspirin-triggered lipoxin in regulating leukocyte-platelet responses in the cerebral microcirculation after ischemia/reperfusion. These results were attained by pharmacological blockade, quantification of endogenous mediator levels, and the use of formyl-peptide receptor 2/3-deficient (Fpr2/3−/−) mice. Furthermore, we demonstrate that specifically neutrophil Fpr2 regulates cerebral neutrophil-platelet aggregation by rapid generation of aspirin-triggered lipoxin in an endogenous biosynthetic circuit. This pathway helps to resolve inflammation and to restore homeostasis. This has been shown for the first time in the brain, and we feel that these data are exciting because they confirm that Fpr2/lipoxin A2 receptor is a therapeutic target for initiating endogenous proresolving, anti-inflammatory pathways after cerebral ischemia/reperfusion injury, which has clear clinical implications in helping to limit neuronal damage after ischemic insults.
Formyl-Peptide Receptor 2/3/Lipoxin A₄ Receptor Regulates Neutrophil-Platelet Aggregation and Attenuates Cerebral Inflammation: Impact for Therapy in Cardiovascular Disease

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Supplemental Methods

Animals

All studies were done blinded and performed on adult male mice weighing 25-30g. Wild-type (WT) C57BL/6 mice were purchased from either Jackson Laboratory (Bar Harbor, ME, USA) or Charles River Limited (Margate, Kent, UK). Fpr2/3−/− mice were backcrossed for over six generations on a C57BL/6 background and bred on site. The Fpr2/3−/− mice showed no obvious phenotype and were fertile. Mice were maintained on a 12 hours (h) light-dark cycle during which room temperature was maintained at 21–23°C, and had access to a standard chow pellet diet and tap water ad libitum. All animal experiments complied with ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines and followed the European Union Directive (2010/63/EU) or approved by the Louisiana State University Health Sciences Center-Shreveport Institutional Animal Care and Use Committee and were in accordance with the guidelines of the American Physiological Society.

Receptor agonists and drug treatment

Compounds or vehicle (saline, or ethanol plus saline for ATL) were administered (100 μl) intravenously (i.v.) at the start of cerebral reperfusion. Doses chosen were based on previous studies, as follows: AnxA1 mimetic peptide Ac2-26 (AnxA1Ac2–26, Ac-AMVSEFLQAWFIENEEQEYVQTVK, Cambridge Research Biochemicals, Cleveland, UK) was given at a dose of 100 μg per mouse;2 Boc2 (N-tert-butoxycarbonyl-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe; MPBiomedicals, Cambridge, UK) was given at a dose of 10 μg per mouse;2 and ATL (stable epimer of LXA4) was given at a dose of 4.0 μg per mouse.1 In a separate set of experiments, mice were intraperitoneally (i.p.) treated with Aspirin (ASA. 150 mg/kg Sigma-Aldrich, Dorset, UK) 60 minutes (min) prior to I/R.
**Middle cerebral artery occlusion and reperfusion (MCAo)**

As a cerebral I/R model, MCAo was performed as previously reported.² Briefly, mice were anaesthetized with i.p. injection of ketamine (150 mg/kg) and xylazine (7.5 mg/kg) and MCA was occluded for 60 min using a 6-nylon intraluminal filament (Doccol Corporation, Massachusetts, USA), followed by 4 h or 24 h of reperfusion. Sham-operated mice were subject to anesthesia and other surgical procedures without MCA occlusion and analyzed 5 h or 25 h after start of surgery.

**Blood sampling and cell count**

Mice were placed under a heat-lamp, and the tip of tail (~1mm) was cut with scissors, and heparinized capillary tubes were used to collect blood samples. Whole blood samples obtained from tail-vein bleed were used for measuring leukocytes (3% citric acid & 10% crystal violet) and platelet (1% buffered ammonium oxalate) counts with a hemocytometer.

**Platelet and leukocyte labeling**

Blood from donor mice was withdrawn in syringes prefilled with 85 mM sodium citrate, 62.2 mM citric acid, and 110 mM glucose (ACD buffer, Sigma-Aldrich). Platelet-rich plasma was obtained by centrifugation (118 × g, 8 min) before platelet isolation by centrifugation at 735 g for 10 min. Platelets were gently resuspended, counted, and labeled by using carboxyfluorescein succinimidyl ester (CFSE, 90 µM, 10 min, Sigma-Aldrich). After confirming absence of aggregates by light microscopy, 100 × 10⁶ platelets in 120 µL saline were injected through the jugular vein of a recipient mouse over 5-minute infusion as previously described.³ This was followed by the continuous infusion of 0.02 % rhodamine 6G, which fluorescently labeled circulating leukocytes.
Cerebral intravital fluorescence microscopy (IVM)

IVM was performed as previously described. Briefly, mice were re-anaesthetized with i.p. injection of ketamine (150 mg/kg) and xylazine (7.5 mg/kg), and the jugular artery and vein cannulated to monitor mean arterial blood pressure (MABP), as well as for i.v. administration of rhodamine 6G and ex vivo labeled platelets. The head of each mouse was then fixed in a plastic frame in sphinx position. The left parietal bone was exposed by a midline skin incision, followed by a craniectomy (diameter: 2.5 mm) with a drill at 1 mm posterior from the bregma and 4 mm lateral from the midline. The dura mater was not cut because the fluorescent-labeled leukocytes were readily visualized. A 12 mm glass coverslip was placed over the craniectomy and the space between the glass and dura mater was filled with artificial cerebrospinal fluid (aCSF; Na⁺ 147.8 mEq/L, K⁺ 3.0 mEq/L, Mg²⁺ 2.3 mEq/L, Ca²⁺ 2.3 mEq/L, Cl⁻ 135.2 mEq/L, HCO₃⁻ 19.6 mEq/L, lactate⁻ 1.67 mEq/L, phosphate 1.1 mM, and glucose 3.9 mM; all Sigma-Aldrich). A Zeiss Axioskop microscope (Zeiss, New York, USA) with a mercury lamp was used to observe the pial venules in the cerebral cortex. A Hitachi charge-coupled device color camera (model KPC571; Tokyo, Japan) acquired images that were recorded for offline analysis. One to five randomly selected vessel, 30–70 μm in diameter and 100 μm long, were observed for each mouse after treatment. Adherent leukocytes and platelets were defined as cells remaining stationary within venules for ≥30 seconds (s) or ≥2 s respectively. These parameters were expressed as the number of cells per square millimeter of the vessel surface and calculated from diameter and length, assuming cylindrical shape. Estimates of shear rate or pseudo-shear rate in cerebral vessels were obtained by fluorescence microscopy based on image analysis determinations of the maximal velocity of fluorescently labeled red blood cells. Such estimates of shear or pseudo-shear rate are obtained using measurements of venular diameter (Dv) and the maximal velocity of flowing red blood cells (Vcell) according to the formulation:

shear or pseudoshear rate = (Vcell/1.6)/Dv × 8.²
Infarct volume

After a 24 h reperfusion period, mice were killed and brains were immediately removed, placed into 4°C phosphate-buffered saline (PBS, Sigma-Aldrich) for 15 min; 2 mm coronal sections were then cut with a tissue cutter. The brain sections were stained with 2 % 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer at 37°C for 15 min and fixed by immersion in 10% formaldehyde. The stained sections were photographed and the digitized images of each brain section (and the infarcted area) were quantified using a computerized image analysis program (NIH Image Software).

Neurological score

The functional consequences of cerebral I/R injury were evaluated by using a five-point neurological deficit score (0, no deficit; 1, failure to extend right paw; 2, circling to the right; 3, falling to the right; and 4, unable to walk spontaneously) and were assessed in a blinded fashion. A maximal score of 4 could be assigned to each experimental animal.

Blood–Brain Barrier (BBB) Dysfunction

BBB permeability was assessed using the Evans blue (EB) extravasation, as described previously. Briefly, a 2 % solution of EB (Sigma-Aldrich) was injected (4 mL/kg) i.v. Twenty-four hours later, blood was obtained for plasma collection and brains were harvested after transcardial perfusion with PBS for 5 min. The cerebral hemispheres and the plasma were homogenized in 50 % trichloroacetic acid, sonicated, and then centrifuged. The supernatant was diluted with ethanol and the concentrations of Evans blue in brain tissue and plasma were measured using a fluorescence spectrophotometer.
BBB permeability was normalized by dividing tissue EB concentration (μg/g brain weight) by the plasma concentration (μg/mL).

**Myeloperoxidase (MPO) activity**

MPO in brain homogenates was measured as a marker for cerebral neutrophil infiltration. Brain homogenates and MPO standards (Sigma-Aldrich) were placed onto a 96-well plate, and 200 μL of o-dianisidine (Sigma-Aldrich) solution and 10 μL of 0.1 % H₂O₂ (Sigma-Aldrich) were added. The absorbance was read after 5 min at 405 nm and expressed as units per mg of wet tissue.²

**Confocal Microscopy**

To visualize NPAs, mice were injected with specific antibodies to label: neutrophils (eFluor 660 (green)-labeled anti-Mouse Ly-6G, eBioscience, San Diego, CA. 2μg/mouse) and platelets (Dylight 649 (red)-labeled anti-mouse CD42, (Emfret Analytics, Eibelstadt, Germany. 1μg/mouse), as previously described.⁵,⁶ Mice were placed on an Olympus BX51WI upright microscope (Olympus, Center Valley, PA) with a 20X (LUCPlanFLN) objective and equipped with a 3i LaserStack laser launch (3i, Denver, CO), Yokogawa CSU-X1-A1N-E spinning disk confocal unit (Yokogawa Electric Corporation, Tokyo, Japan) and electron multiplier CCD camera (C9100-13, Hamamatsu, Bridgewater, NJ), in order to visualize NPAs. Slidebook software (3i) was used to drive the confocal system and capture images.⁷

**Cytokines in Plasma and Brain Tissue**

24 h after reperfusion, blood was collected into heparin-coated syringe, and plasma was obtained. After a 5 min transcardial perfusion with PBS, brain hemispheres were homogenized, sonicated, and centrifuged in PBS containing protease inhibitors (Bio-Rad, California, USA). Levels of the pro- and
anti-inflammatory cytokines: interleukin-1β (IL-1β), tumor necrosis factor alpha (TNFα), monocyte chemoattractant protein-1 (MCP-1 or CCL2), IL-10 and IL-6, were measured using standard ELISAs (Quantikine™ immunoassay kits, R&D Systems, Abingdon, UK). ATL and thromboxane B2 (TXB2) concentrations in plasma and brain homogenates were determined using ELISA kits from Neogen and GE Healthcare, respectively. We followed the manufacturer’s specifications for lipid extraction. Cytokine concentrations were expressed as either pg/ml (plasma) or pg/g brain weight (brain).

**Assessment of activated, immature and mature platelets**

Murine blood obtained by tail-vein bleed before and after MCAo was mixed with heparin (20U/mL), as described previously. Briefly, platelets were identified by their characteristic light scattering and membrane expression of the specific platelet glycoprotein IIb (CD41) detected with rat anti-mouse CD41-APC antibody (eBiosciences, Inc). Two-color staining of JON/A-PE (Emfret Analytics, Wurzburg, Germany) and thiazole orange (TO, Sigma-Aldrich, St Louis, MO) was used. Platelet activation was assessed by the binding of the JON/A-PE antibody to the activation epitope of GPIIb/IIIa and expressed as mean fluorescent intensity (MFI). Appropriate rat IgGs were used to determine non-specific binding. Immature platelets were identified using TO (1μg/mL dissolved in PBS). Fresh blood was diluted 1:5 and stained for 15 min at 20°C and analyzed with a LSRII flow cytometer (BD Biosciences, San Jose, CA). 20,000-50,000 events were collected, and the data were analyzed using FACSDiva software (BD Biosciences, San Jose, CA). The immature platelet population was identified by setting a TO-high gate that is 5% of the total platelet population, as previously described.

**Statistical Analysis**
Results from IVM experiments were confirmed to follow a normal distribution using Kolmogorov-Smirnov test of normality with Dallal-Wilkinson-Lillie for corrected $p$ value. Data that passed the normality assumption was analyzed using Student’s $t$-test (two groups: Figures 3A-E, 5A+B, 5E-H) or ANOVA with Bonferroni post-tests (more than two groups: Figures 1A,B, 2B-D, 3F-J, 4A, 4C+D, 5C+D, supplementary Figure 2A-D), which were performed using GraphPad Prism5 software. Data that failed the normality assumption were analyzed using the non-parametric Mann-Whitney U test (two groups: Table 1 [total WBC]) or Kruskal-Wallis with Dunn’s test (more than two groups: Table 1, Figure 2A, Supplementary Figure 1A-D). Data are shown as mean values ± standard error of the mean (SEM), or median with interquartile range (neurological score only). Differences were considered statistically significant at a value of $p < 0.05$. 
Supplemental References


Supplemental Figure Legends

Supplemental Figure 1. Number of peripheral immature and mature platelets and levels of activation within these populations in WT and Fpr2/3 mice, pre and post stroke. Blood samples were taken from wild type (WT, C57BL/6) and Fpr2/3−/− mice, before and after MCAo (60 min, followed by 24 h reperfusion). A) The appearance of immature (identified as TO+ (TO high) platelets) and B) mature platelets (identified as TO− (TO low) platelets) were quantified, and the platelet populations were gated on CD41+ events. Circulating mature and immature activated platelets were identified as JONA+ platelets. C) Activated immature platelets (TO+ JONA+) and D) activated mature platelets (JONA+/TO−), expressed as mean fluorescent intensity (MFI). Data are mean ± SEM of 4-6 mice per group.

Supplemental Figure 2. Exogenous Fpr2/3 agonists reduce I/R-induced cerebrovascular injury. Wild type (WT, C57BL/6) and Fpr2/3−/− mice were subjected to MCAo for 60 min, followed by 24 h reperfusion. Vehicle (V. saline) or Agonists: AnxA1 Ac2–26 (100 μg per mouse) and ATL (stable epimer of LXA4. 4.0 μg/mouse); antagonist: Boc2 (10 μg per mouse) or a combination (i.e. AnxA1 Ac2–26 (100 μg per mouse) + Boc2 (10 μg per mouse); ATL (4.0 μg per mouse) + Boc2 (10 μg per mouse)) were given at the start of reperfusion. Leukocyte and platelet recruitment in the cerebral microcirculation was quantified in terms of: C) number of adherent leukocytes (i.e. those cells stationary for 30 sec or longer) and D) number of adherent platelets (i.e. those cells stationary for 2 sec or longer). E) Infarct volume and D) Neurological score were also assessed. Data are mean ± SEM of 6-8 mice per group. *p < 0.01 & ***p < 0.001 vs. vehicle. #p < 0.05 & ###p < 0.001 vs. corresponding Ac2-26 or ATL alone.
**Supplemental Table 1. Hemodynamic parameters of WT and Fpr2/3^-/- mice.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body weight (g)</th>
<th>Treatment</th>
<th>Vessel diameter (µm)</th>
<th>pseudo-shear rate (per s)</th>
<th>Mean arterial blood pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>28.5 ± 0.6</td>
<td>Sham</td>
<td>35.8 ± 2.2</td>
<td>599 ± 85</td>
<td>78.3 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>29.5 ± 0.3</td>
<td>I/R</td>
<td>38.2 ± 1.0</td>
<td>489 ± 59</td>
<td>82.1 ± 3.9</td>
</tr>
<tr>
<td>Fpr2/3^-/-</td>
<td>29.1 ± 0.4</td>
<td>Sham</td>
<td>37.1 ± 1.8</td>
<td>601 ± 72</td>
<td>80.3 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>29.5 ± 0.5</td>
<td>I/R</td>
<td>36.2 ± 1.4</td>
<td>470 ± 102</td>
<td>83.1 ± 5.4</td>
</tr>
</tbody>
</table>

I/R = 60 min ischemia + 24 h reperfusion.
Supplemental Figure 1
Supplemental Figure. 2