Systemic Inflammation Impairs Tissue Reperfusion Through Endothelin-Dependent Mechanisms in Cerebral Ischemia

Katie N. Murray, BA (Hons); Sylvie Girard, PhD; William M. Holmes, PhD; Laura M. Parkes, PhD; Stephen R. Williams, PhD; Adrian R. Parry-Jones, PhD, MRCP; Stuart M. Allan, PhD

Background and Purpose—Systemic inflammation contributes to diverse acute and chronic brain pathologies, and extensive evidence implicates inflammation in stroke susceptibility and poor outcome. Here we investigate whether systemic inflammation alters cerebral blood flow during reperfusion after experimental cerebral ischemia.

Methods—Serial diffusion and perfusion-weighted MRI was performed after reperfusion in Wistar rats given systemic (intraperitoneal) interleukin-1β or vehicle before 60-minute transient middle cerebral artery occlusion. The expression and location of endothelin-1 was assessed by polymerase chain reaction, ELISA, and immunofluorescence.

Results—Systemic interleukin-1 caused a severe reduction in cerebral blood flow and increase in infarct volume compared with vehicle. Restriction in cerebral blood flow was observed alongside activation of the cerebral vasculature and upregulation of the vasoconstricting peptide endothelin-1 in the ischemic penumbra. A microthrombotic profile was also observed in the vasculature of rats receiving interleukin-1. Blockade of endothelin-1 receptors reversed this hypoperfusion, reduced tissue damage, and improved functional outcome.

Conclusions—These data suggest patients with a raised inflammatory profile may have persistent deficits in perfusion after reopening of an occluded vessel. Future therapeutic strategies to interrupt the mechanism identified could lead to enhanced recovery of penumbra in patients with a heightened inflammatory burden and a better outcome after stroke. (Stroke. 2014;45:00-00.)

Key Words: brain ischemia ■ endothelin-1 ■ inflammation ■ interleukin-1 ■ magnetic resonance imaging ■ reperfusion

A raised systemic inflammatory profile is observed in comorbidities associated with vascular disease, including hypertension, infection, atherosclerosis, obesity, and diabetes mellitus.1–3 Observational clinical studies show that inflammation is independently associated with an increased risk of stroke as well as poor poststroke outcomes.4,5 Robust preclinical evidence shows that systemic inflammation markedly exacerbates acute brain injury,6,7 but the underlying mechanisms remain unclear. The proinflammatory cytokine interleukin-1 (IL-1) is a major driver of inflammation, and the detrimental effects of systemic IL-1 are well documented in multiple preclinical models of systemic inflammatory disease as well as in cerebral ischemia.8–10

Rapid restoration of cerebral blood flow (CBF) after acute ischemia, either spontaneously or through thrombolysis, reduces tissue damage. However, the benefit of reperfusion falls within a few hours of onset of ischemia, eventually becoming potentially harmful if timely reperfusion is not achieved.11 Recanalization is an established reperfusion therapy for acute stroke;12 however, human imaging studies have shown that when recanalization is achieved it does not necessarily lead to restoration of tissue blood flow.13 Restoration of CBF is likely to be more important than recanalization, and consistent with this, tissue reperfusion has been shown to be a better predictor of recovery from stroke than recanalization.14 Pre-existing systemic inflammation may influence mechanisms needed to sustain adequate perfusion after stroke. Therefore, we have used a previously validated model15 of systemic inflammation to test the hypothesis that upregulation of vasoactive mediators may contribute to the observed association between inflammation and poor outcome after cerebral ischemia. We show that systemic IL-1 markedly increases the extent of tissue hypoperfusion after reperfusion; increased expression of endothelin-1 (ET-1) accompanies this hypoperfusion and that blockade of this mechanism results in restoration of CBF, reduced infarct, and an improved outcome after experimental stroke.

Materials and Methods

More detailed methods are available in the online-only Data Supplement.
Animals
All experiments were performed using male, 9-week-old Wistar rats (350–450 g; Charles River Laboratories) and adhered to the Animals (Scientific Procedures) Act, UK (1986).

Focal Cerebral Ischemia
Focal cerebral ischemia was induced by transient (60 minutes) middle cerebral artery occlusion (MCAo). A, Representative apparent diffusion coefficient (ADC) maps with hypoperfused volume (red) and infarct core (black) overlaid, highlighted the mismatch between infarct (DWI) and cerebral blood flow deficit (PWI) at 15 min, 2 h, and 4 h after reperfusion in vehicle (top) and IL-1β–treated animals (bottom). Cresyl violet staining shows infarct at 24 h in vehicle and IL-1β–treated rats. Acute evolution of perfusion deficit (B) and ADC-derived ischemic lesion volume (C) in vehicle and IL-1β–treated animals after 60 min MCAo (2-way repeated-measures ANOVA, Bonferroni post-test). Data are presented as mean±SD (n=8), ***P<0.001, **P<0.01, *P<0.05.

Figure 1. The effects of systemic inflammation on cerebral blood flow. Systemic inflammation was induced by intraperitoneal interleukin-1 (IL-1β), and effects on perfusion deficit and infarct evolution using diffusion-weighted imaging and perfusion weighted (DWI-PWI) were assessed after transient middle cerebral artery occlusion (MCAo). A, Representative apparent diffusion coefficient (ADC) maps with hypoperfused volume (red) and infarct core (black) overlaid, highlighted the mismatch between infarct (DWI) and cerebral blood flow deficit (PWI) at 15 min, 2 h, and 4 h after reperfusion in vehicle and IL-1β–treated animals. B and ADC-derived ischemic lesion volume in vehicle and IL-1β–treated animals after 60 min MCAo (2-way repeated-measures ANOVA, Bonferroni post-test). Data are presented as mean±SD (n=8), ***P<0.001, **P<0.01, *P<0.05.

Treatment
Treatment allocations were randomized and blinded until completion of analysis. Rat recombinant IL-1β (4 μg/kg; National Institute for...
Biological Standards and Controls) or vehicle (0.5% endotoxin-free bovine serum albumin in sterile PBS) was administered intraperitoneally at the onset of surgery, and an intravenous dose of 1 mg/kg of the selective ET-1 receptor A (ETrA) antagonist BQ-123 (Merck Chemicals Ltd) was administered at occlusion and reperfusion. BQ-123 was chosen to ablate the activation of ETrA because of its potent and specific inhibition of ET-1 contractility in rat vascular smooth muscle.17

Assessment of Neurological Deficit
Neurological status and motor function were assessed blinded to drug treatment and according to the 28-point neuroscore, modified from methods previously described.18

Magnetic Resonance Imaging
MRI was performed using a 7-T horizontal bore magnet (Agilent). MR angiography was used to assess occlusion and reopening of the middle cerebral artery. Lesion volume was determined from apparent diffusion coefficient (ADC) maps. Perfusion weighted imaging was performed using pseudocontinuous arterial spin labeling.19,20

MRI Analysis
Quantitative ADC maps were generated in Paravision 5 software (Bruker, Germany) from diffusion-weighted images. A 23% reduction relative to mean contralateral ADC was used as the threshold to define the ischemic lesion21 (Java; General Public License), and a 57% reduction relative to mean contralateral CBF was set as a threshold for hypoperfused tissue.22 Physiological measurements were analyzed using MATLAB (MathWorks Inc).

Histological Analysis
The volume of ischemic damage and hemorrhagic transformation was measured by staining coronal sections (360 μm interval) with cresyl violet (Sigma) and hematoxylin & eosin.
RNA Extraction and Quantitative Polymerase Chain Reaction

Total RNA was extracted from isolated striatum and cortex brain homogenate using Trizol (Life Sciences) following the manufacturer’s instructions. Detailed methods for the quantitative polymerase chain reaction are described elsewhere.23

Enzyme-Linked Immunosorbant Assay

ET-1 concentrations were determined by ELISA (R&D Systems). Absorbance was measured using a plate reader (MRX; Dynatech) at room temperature, and results were calculated from the standard curve using Prism 6 software (GraphPad).

Immunofluorescence

Free-floating coronal brain slices (30 μm) were processed after transient MCAo, and immunofluorescence, microscopy, and image analysis was performed as described in the online-only Data Supplement.

Statistics

Group sizes (n=6–8) were calculated based on previous data. Data are presented as mean±SD. Student t test was used for single comparisons and 1-way or 2-way ANOVA was followed by Bonferroni correction for multiple comparisons or Kruskal–Wallis test followed by Dunn test for multiple comparisons using Prism Graph 6.0 software (GraphPad). Differences were considered significant when P<0.05.

Results

Acute Systemic Inflammatory Challenge Lowered CBF and Worsened Ischemic Damage

Systemic inflammation induced by peripheral administration of IL-1β resulted in larger infarct volumes at 24 hours (vehicle, 66±19 mm³; IL-1β, 166±32 mm³; *P<0.05; Figure 1A). IL-1β–treated animals had a 3-fold higher volume of hypoperfused tissue 15 minutes after reopening the middle cerebral artery, versus vehicle (vehicle, 22±7 mm³; IL-1β, 70±30 mm³; ***P<0.001; Figure 1B). The volume of hypoperfused tissue in IL-1β–treated animals remained significantly higher up to 2 hours after MCA reopening. Treatment with IL-1β did not alter CBF in sham-treated animals (data not shown). ADC-derived lesion volumes were significantly higher (2-fold) in IL-1β–treated compared with vehicle-treated rats at 3 and 4 hours reperfusion (**P<0.01; Figure 1C).

Upregulation of ET-1 mRNA and Protein Was Associated With Larger Perfusion Deficits in Animals With Systemic Inflammatory Challenge

Of the vasoactive mediators studied (Figure I in the online-only Data Supplement), a 2-fold increase in ET-1 mRNA expression was observed in the ipsilateral striatum (***P<0.01) and cortex (*P<0.05) of stroke plus IL-1β versus sham- and vehicle-treated counterparts (Figure 2A and 2B). A 2-fold increase (*P<0.05) in protein expression of ET-1 was measured in the cortex of IL-1β–treated MCAo animals (Figure 2C and 2D).

Figure 4. BQ-123 reverses hypoperfusion induced by systemic inflammation. BQ-123 was used to antagonize endothelin-1 in rats receiving interleukin-1β (IL-1β) or vehicle. A, Representative apparent diffusion coefficient (ADC) maps with hypoperfused volume (red) and infarct core (black) overlaid demonstrating diffusion-weighted imaging and perfusion-weighted imaging mismatch at 15 min, 2 h, and 4 h after reperfusion in vehicle, IL-1β, vehicle plus BQ-123, and IL-1β plus BQ-123–treated animals. Volume of hypoperfusion (B) and ADC lesion volumes (C) were determined at each time point and compared between groups (2-way repeated-measures ANOVA, Bonferroni post test). Data are presented as mean±SD (n=8). ***P<0.001, **P<0.01, *P<0.05 in reference to stroke plus IL-1β vs stroke plus vehicle, ###P<0.001 in reference to stroke plus IL-1β plus BQ-123 vs stroke plus IL-1β.
ETrA is localized to smooth muscle in areas of perfusion deficits. Phosphorylated serine\(^{19}\) on myosin light chains (an integral step in brain endothelial cytoskeletal reorganization) and \(\alpha\)-smooth muscle actin were colocalized in cortical arteries of IL-1\(\beta\)–treated animals at 15 minutes and 4 hours after cerebral ischemia alone (Figure 3A, arrowhead).

ET-1 (Figure 3B, closed arrowhead) was identified in animals receiving IL-1\(\beta\) before cerebral ischemia at both 15 minutes and 4 hours after reperfusion, but in vehicle-treated animals, there was no ET-1 staining at 15 minutes and minimal ET-1 staining at 4 hours. Vessels that stained positive for ET-1 also expressed ETrA and were localized to the ipsilateral hemisphere of IL-1\(\beta\)–treated animals (Figure II in the online-only Data Supplement).

**Inhibition of ETrA Prevented IL-1\(\beta\)–Induced Hypoperfusion**

A diffusion-weighted imaging and perfusion-weighted imaging mismatch was seen 15 minutes, 2 hours, and 4 hours after MCAo in animals receiving vehicle, IL-1\(\beta\), vehicle plus BQ-123 or IL-1\(\beta\) plus BQ-123 (Figure 4A). Animals treated with IL-1\(\beta\) had a significantly larger perfusion deficit at 15 minutes (vehicle, 33±11 mm\(^3\); IL-1\(\beta\), 73±26 mm\(^3\); **\(P<0.001\)**). In contrast, in animals treated with IL-1\(\beta\) plus BQ-123, the volume of hypoperfusion was reduced to levels seen in the vehicle-treated group (IL-1\(\beta\), 73±26 mm\(^3\); IL-1\(\beta\) plus BQ-123, 38±15 mm\(^3\); **\(P<0.001\)**; Figure 4B).

ADC-derived lesion volume was larger in IL-1\(\beta\)–treated than in vehicle-treated rats at 4 hours reperfusion (vehicle, 26±10 mm\(^3\); IL-1\(\beta\), 60±34 mm\(^3\); **\(P<0.001\)**). However, in animals treated with IL-1\(\beta\) plus BQ-123, the volume of infarction was markedly reduced at 4 hours reperfusion (IL-1\(\beta\), 73±26 mm\(^3\); IL-1\(\beta\) plus BQ-123, 38±15 mm\(^3\); **\(P<0.001\)**), such that volumes were not significantly different to vehicle-treated animals (Figure 4C).

**Blockade of ETrA Reduced Ischemic Damage and Improved Behavioral Outcomes**

Systemic IL-1\(\beta\) challenge before MCAo exacerbated the extent of ischemic brain injury (3-fold) and functional deficits at 48 hours reperfusion (Figure 5A and 5B). Conversely, administration of BQ-123 in the presence of IL-1\(\beta\) and MCAo markedly attenuated (4-fold) ischemic damage and behavioral deficits (Figure 5C). The incidence of hemorrhagic transformation (Figure 5D) was not significantly increased in animals receiving BQ-123 (numbers of animals presenting with hemorrhagic transformation: vehicle, n= 2/6; IL-1\(\beta\), n=1/6; vehicle plus BQ-123, n= 2/6; IL-1\(\beta\) plus BQ-123, n=1/7).

**Acute Systemic Inflammatory Challenge Lowered CBF and Was Associated With a Prothrombotic State**

At 4 hours reperfusion, platelet CD41-positive immunostaining was seen within activated vessels of animals treated with
systemic IL-1β (Figure 6A). Presence of IL-1β was associated with significant accumulation of platelets (vehicle, 11±7 per mm²; IL-1β, 52±15 per mm²; **P<0.01) and formation of small aggregates (vehicle, 10±8 per mm²; IL-1β, 44±31 per mm²; *P<0.05; Figure 6B) in the cortex of IL-1β–treated animals at 4 hours reperfusion. Treatment with BQ-123 did not completely deplete the number of platelets adhering to the endothelium at 4 hours reperfusion, although a trend toward reduction in platelet numbers was seen compared with IL-1β alone (IL-1β, 124±57 per mm²; IL-1β plus BQ-123, 61±37 per mm²; P=0.068; Figure 6C). Blockade of ET-1 significantly reduced platelets in vehicle plus BQ-123–treated animals (*P<0.05) and reduced platelet aggregates of <10 and 10 to 30 μm² in vehicle plus BQ-123– and IL-1β plus BQ-123–treated animals at 4 hours reperfusion in the cortex (*P<0.05).

Discussion

We have shown that systemic IL-1β has a crucial role in limiting blood flow to metabolically compromised brain tissue, and upregulation in ET-1 plays an important role in this process despite recanalization. Restoration of CBF during acute reperfusion was achieved through ETra antagonism, leading to improved tissue survival and functional outcomes. Clinical and experimental studies show an association between inflammation and a higher risk of ischemic stroke as well as worse poststroke outcome.24,25 Elevated IL-1β is a central component of these pathologies as seen in chronic and acute inflammatory models2,26; thus, administration of systemic IL-1β is a relevant model for many conditions that predispose to stroke. It is thought that much of the detrimental actions of IL-1β on ischemic stroke are exerted peripherally as levels of IL-1β are undetectable in the brain.10 Furthermore, IL-1β has been shown to affect the magnitude of the acute phase response, which is a key indicator of peripheral inflammation. IL-1β causes an increase in C-reactive protein and IL-6, and in both preclinical10 and clinical studies,27 raised levels of these markers are associated with poor prognosis. Peripherally administered lipopolysaccharide, which mimics aspects of infection, has also been used to represent acute inflammation before stroke, and mice treated with lipopolysaccharide just before stroke show a marked increase in ischemic injury, an effect that is inhibited by administration of the IL-1 receptor antagonist.10 Our recent data show that preceding pneumonia infection in mice also worsens ischemic brain damage, an effect dependent again on IL-1β as well as platelet–endothelial interactions.28 This suggests endogenous IL-1β has a key role in exacerbation of ischemic damage by systemic inflammation, most likely through effects on the cerebrovasculature. Clinical relevance of these experimental findings is demonstrated by the finding that patients with pneumonia develop systemic cytokine responses and show raised levels of circulating IL-1β concentrations.29,30 Previous studies have reported either no effect on blood vessel dynamics or a rise in cerebral blood volume in response to IL-1β, but in both studies, IL-1β was administered in the absence of injury and it is likely that IL-1β acts differently in the presence of ischemia.31,32 Our finding that ET-1 was upregulated only in animals exposed to both cerebral ischemia and an elevated inflammatory status and not in sham-treated animals lends further support to this hypothesis.
Smooth muscle cells have a critical role in regulating vascular tone in response to vasoactive mediators released by the endothelium and neighboring cells. Previous experimental studies have shown the potent vasoconstrictive effects of ET-1 through its actions on ETRα on smooth muscle cells, in contrast to the predominantly vasodilatory ET-1 receptor B that is abundant on endothelium and neurons. Both ETRα and ET-1 receptor B are upregulated after injury, but only blockade of ETRα before injury prevents hypoperfusion. Although antagonism of ETRα restored CBF and reduced ischemic damage in animals with a pre-existing inflammatory challenge, infarct was not reduced in stroke plus BQ-123–treated animals as seen in previous studies. It is possible this discrepancy was because of the negligible volume of penumbral tissue remaining (as indicated by the diffusion-weighted imaging and perfusion-weighted imaging mismatch) for salvaging by BQ-123. Furthermore, the observed increase in ET-1 expression and vessel contractility occurred primarily in the cortex of IL-1β–treated animals. This colocalizes with tissue at risk of infarction and suggests that endothelin antagonism rescues penumbral tissue in the cortex rather than the necrotic infarct core of the striatum.

Despite differences in disease progression, hypoperfusion in both chronic and acute inflammatory conditions has been established in the experimental and clinical setting. In patients with multiple sclerosis, an increase in plasma ET-1 was noted alongside a corresponding reduction in CBF as measured by perfusion-weighted imaging, whereas postmortem studies show an upregulation of ET-1 in the brain parenchyma in multiple sclerosis. The vasoconstrictive actions of endothelium-derived ET-1 on smooth muscle are well elucidated, but here we report that the presence of systemic IL-1β acts as a trigger for vessel contractility via ET-1 after cerebral ischemia. It is possible that IL-1β activates the endothelium to increase production of ET-1 because both ischemic stroke and IL-1 are potent activators of the cerebrovasculature, and endothelial cells are a source of ET-1. Although we cannot rule out that other vasoactive mediators might contribute to reductions in CBF in the presence of systemic inflammation, our data do support an important, selective role for ET-1.

Our findings may have clinical implications beyond representing a mechanistic explanation for the worse outcome observed in stroke patients with a pre-existing elevated inflammatory status. Currently, recanalization represents a strong predictor of stroke outcome and is being increasingly used as a surrogate marker for efficacy in thrombolytic and other recanalization trials in acute stroke. However, we have shown that an elevated systemic inflammatory profile can result in hypoperfusion and subsequent excess ischemic damage. This was prevented by BQ-123, therefore suggesting that recanalization does not necessarily lead to brain tissue reperfusion and that an elevated inflammatory status may be an important complimentary therapeutic target for patients with stroke.

In conclusion, we have demonstrated that systemic inflammation may worsen outcomes after cerebral ischemia by IL-1β–induced hypoperfusion in the presence of ET-1 during early reperfusion. This reduction in CBF leads to larger infarcts and worse functional outcomes despite large vessel recanalization. Interrupting this mechanism pharmacologically may improve recovery of CBF after thrombolysis or intra-arterial recanalization in patients with a high peripheral inflammatory burden, potentially improving outcomes after clinical stroke.

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Disclosures
None.

References


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Supplemental material

Systemic inflammation impairs tissue reperfusion through endothelin-dependent mechanisms in cerebral ischemia

Katie N Murray, Sylvie Girard, William M Holmes, Laura M Parkes, Stephen R Williams, Adrian R Parry-Jones, Stuart M Allan.

Supplementary methods

Animals: All experiments were performed using male, 9-week old, Wistar rats (350-450g) (Charles River Laboratories, UK) under appropriate United Kingdom Home Office licenses and all procedures adhered to the Animals (Scientific Procedures) Act (1986).

Focal cerebral ischemia: Focal cerebral ischemia was induced by transient (60min) middle cerebral artery occlusion (MCAo) using a modification of the intraluminal filament model originally described by Longa et al. Core body temperature was regulated at 37°C±1°C by a homeothermic blanket (Harvard Apparatus, UK). Anesthesia was induced (4%) and maintained (1.5–2%) with isoflurane delivered in 70:30 N2O/O2. The bifurcation of the carotid artery was exposed and a 30mm long, 0.19mm diameter suture with a silicon coated tip measuring 350±20µm in diameter and 5-6mm in coating length monofilament (Doccol Corporation, USA) was advanced along the internal carotid artery (ICA) until occlusion of the MCA. The filament was withdrawn to establish reperfusion, monitored using Laser Doppler flowmetry (Oxford Optronix, UK). Sham-operated animals underwent the same procedure except the filament was advanced to the MCA, and then immediately withdrawn. Mortality rate was <15% in all groups except where stated. The femoral artery was cannulated for continuous monitoring of arterial blood pressure (Fluid-filled pressure transducer, AD Instruments, UK), blood gas analysis and blood sampling. Heart rate, respiratory rate and temperature were continuously measured during the reperfusion period (ECG trigger unit, Rapid Biomedical, UK).

Animals were excluded from statistical analysis based on an a priori exclusion criterion, namely if Laser Doppler flowmetry did not indicate a drop in CBF of >60% at the point of occlusion, if magnetic resonance angiography did not show full restoration of CBF following remote reperfusion and in extremes of physiological measurements. Subarachnoid hemorrhage was not observed in any groups in any experiments. Hemorrhagic transformation was observed in 6 animals in total as detailed in figure 5D. Of the 128 animals undergoing tMCAo, 16 rats met the a priori exclusion criteria during imaging/occlusion period prior to recovery and were withdrawn from the study (the groups they occupied were: stroke + veh: 6; stroke + IL-1β: 5; stroke + veh + BQ-123: 2; stroke + IL-1β + BQ-123: 3). 3 animals were excluded from the studies following recovery from anesthesia from each of the following groups: stroke + veh (labored breathing and non-responsive), stroke + IL-1β (labored breathing and non-responsive), stroke + veh + BQ-123 (experienced seizure due to cortical infarct). Of the 3 data points excluded from PCR analysis in figure 2 (groups: sham + IL-1β and 2 from stroke + IL-1β), this was due to mRNA contamination as measured by nanodrop values. Of the 3 animals excluded from protein analysis (groups: sham + veh, stroke + veh
and stroke + IL-1β), this was due to insufficient removal of protein debris which interfered with the assay. For the purpose of the imaging experiments, group sizes were determined by power calculation ($\alpha=0.05$, $\beta=0.2$) assuming a group difference of 40% and a SD of 30%.

**Assessment of neurological deficit:** Neurological status and motor function was assessed blinded to drug treatment and according to the 28-point neuroscore which was modified from methods previously described$^2$. The 28 point neuroscore uses 11 tests with a cumulative maximum score of 28: (1) circling (maximum 4 points), (2) motility (maximum 3 points), (3) general condition (maximum 3 points), (4) righting reflex when placed on back (maximum 1 point), (5) paw placement of each paw onto a table top (maximum 4 points), (6) ability to pull self up on a horizontal bar (maximum 3 points), (7) climbing on an inclined platform (maximum 3 points), (8) grip strength (maximum 2 points), (9) contralateral reflex (maximum 1 point), (10) contralateral rotation when held by the base of tail (maximum 2 points), and (11) visual forepaw reaching (maximum 2 points). Scoring was determined on a scale starting from 0 for severe impairment to the maximum score for healthy function$^3$.

**MRI:** Magnetic resonance imaging (MRI) was performed on a 7T horizontal bore magnet (Agilent, UK) interfaced to a BrukerAvance III console (Bruker, UK) with separate volume-transmit and surface-receive radiofrequency coils. Coronal pilot images were acquired to determine the correct geometry (using a multi-slice gradient echo sequence). Magnetic resonance angiography was used to assess occlusion and re-opening of the MCA (TR = 15ms; TE = 3.8ms; flip angle [FA] = 80°; matrix size = 256x256; FOV = 4cm. 0.4mm slice thickness; 0.25mm interslice distance; acquisition time = 11min 31s). Diffusion weighted images (DWI) were obtained using a spin echo sequence (TE = 18.5ms; TR = 1800ms; 1 average; matrix = 96x96; FOV = 4cm; 3 directions = x, y, z; b values = 0 and 1000s/mm$^2$; 9 contiguous slices; 2mm slice thickness; acquisition time = 5min 52s) to determine lesion volume by calculating apparent diffusion coefficient (ADC) maps. Perfusion weighted imaging (PWI) was obtained using a pseudo-continuous arterial spin labeling sequence employing a train of 47 adiabatic inversion pulses over 3000ms followed by an EPI read-out$^4$. 3 contiguous 2mm slices were selected spanning the MCAo territory (TE = 15.37ms; TR = 2500ms; 6 averages; matrix = 96x96; FOV = 4cm; 2mm slice thickness; 2mm interslice distance; 40s acquisition time; EPI flip angle = 90°) and there was a 2cm offset between the labeling and imaging slice$^4$.$^5$. DWI and PWI scans were performed every hour for 4 hours, post-reperfusion. Use of DWI-PWI allowed assessment of the temporal and spatial evolution of CBF changes immediately following reperfusion in animals with a systemic inflammatory challenge and correlation with infarct progression$^6$.

**MRI Analysis:**
Co-aligned images from DWI and ASL imaging were used to generate quantitative ADC maps and CBF maps respectively. Quantitative ADC maps were generated in Paravision 5 software (Bruker, Germany). A 23% reduction relative to mean contralateral ADC was used as the threshold to define the ischemic lesion and lesion volume was determined from multislice ADC maps$^7$ using Image J (Java, General public license). A 57% reduction relative to mean contralateral CBF was set as a threshold for hypoperfused tissue$^8$ with the volume of hypoperfused tissue measured with Image J. Thresholds for acute perfusion lesion and tissue at risk of infarction were previously identified and ASL has been shown to have similar accuracy to PWI and CT perfusion-Tmax thresholds$^9$. Negative perfusion values were generated in some pixels with low or zero flow as a consequence of subtracting the
magnitude images ($S^{\text{control}}$ and $S^{\text{Label}}$), where the signal difference was comparable to image noise. Negative ASL CBF values were assumed to be zero. Physiological measurements were analyzed using MATLAB (MathWorks Inc, USA).

**Tissue processing:** For immunohistochemistry, rats were transcardially-perfused with 0.9% saline followed by 4% paraformaldehyde or 0.1% Diethyl Pyrocarbonate (DEPC) treated sterile saline. Brains were removed, post-fixed, cryoprotected, and frozen or snap frozen with isopentane (Sigma, UK). For all experiments, sections (30µm) were cut on a sledge microtome (Bright Series 8000, UK).

**RNA Extraction and quantitative reverse transcriptase PCR:** Total RNA was extracted from isolated striatum and cortex brain homogenate using Trizol (Life Sciences, UK) following the manufacture’s instructions. RNA was reverse transcribed using MMLV RT, oligo-dT, RNase out, and dNTPs (Life Sciences, UK). Detailed methods for the qPCR were previously described. All primers were from Qiagen (Quantitect Primers Assay, UK), and a fast real-time PCR system (7900 HT, Applied Biosystem, UK) was used. Rat mRNA for ET-1, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin-E$_2$ (PGE$_2$) and P-selectin were analyzed due to their vasoactive and thrombotic capacity as detailed previously. The expression of two housekeeping genes was also analyzed (i.e. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase complex, subunit A (sDHA). No significant differences were observed between the housekeeping genes (data not shown). The relative gene expression of the phenotype markers was normalized to sDHA, and the levels of expression in each of the conditions determined as fold increase compared with vehicle-treated sham animals.

**Immunofluorescence:** Free-floating sections (30µm thick) were washed in PBS and incubated for 60min in blocking solution (PBS containing 5% donkey serum, Serotec, UK), 0.5% bovine serum albumin and 0.5% Triton X-100). Sections were then incubated overnight at 4°C in blocking solution with primary antibody (Ab) including rat anti-CD41 (BD Bioscience, UK); goat anti-intercellular adhesion molecule 1 (ICAM-1)(R&D System, UK); mouse anti-alpha smooth muscle actin (Sigma, UK); rabbit anti-ET-1 (Biorbyt, UK); rabbit anti-ET-1 receptor A (Abcam, UK); rabbit anti phospho-myosin light chain 2 (pMLC) (Cell Signaling Technology, UK); rabbit anti-neutrophil serum (SJC) kindly provided by Drs. Daniel Anthony and Sandra Campbell, University of Oxford. Sections were washed in PBS and incubated with secondary Ab (anti-rabbit Alexa-594; anti-goat Alexa-488; anti-rat Alexa-594; anti-mouse Alexa 488; Life Sciences, UK) for 3h. Sections were washed with PBS, mounted onto slides and cover slipped with Prolong anti-fade medium (Life Sciences, UK).

**Cell counts:** Total numbers of cells in brain tissue sections were determined via wide field microscopy on an Olympus BX51 upright microscope (Olympus, UK) and captured using MetaVue software (Molecular Devices, USA) following immunohistochemistry staining. Cells were quantified in a blinded manner by counting the number of CD41-immunopositive cells in three areas of the cortex or striatum at 5 coronal levels. The mean was calculated from the 6 fields and adjusted to express as mean number of cells/mm$^2$ in the predefined brain region. To further measure cell aggregation, the area of individual cells/cluster of cells was calculated and categorized as small (2–5 cells), medium (5–10 cells), or large (>10 cells).
Supplementary table I.

Table I. Physiologic parameters of vehicle and IL-1β measured from 1 to 4h reperfusion.

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<td>105±14</td>
<td>103±11</td>
<td>100±8</td>
<td>96±9</td>
</tr>
<tr>
<td><strong>PaO₂ (mm Hg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>140±6</td>
<td>144±3</td>
<td>138±5</td>
<td>141±1</td>
<td>140±5</td>
</tr>
<tr>
<td>IL-1β</td>
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<td>142±2</td>
<td>143±0.5</td>
<td>108±54</td>
<td>144±2</td>
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<tr>
<td><strong>PaCO₂ (mm Hg)</strong></td>
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<td></td>
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<tr>
<td>Vehicle</td>
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<td>28±9</td>
<td>27±12</td>
<td>40±3</td>
<td>28±6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>36±7</td>
<td>31±4</td>
<td>44±12</td>
<td>50±14</td>
<td>27±8</td>
</tr>
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</table>

Supplementary Table I: Physiological parameters were monitored throughout the surgical and imaging procedures. Time course changes of temperature; MABP - mean arterial blood pressure; PaO₂, partial pressure of oxygen and PaCO₂, partial pressure of carbon dioxide following intraperitoneal injection of either vehicle or IL-1β following 60min MCAo (n=7). Temperature was maintained at 37 ±1°C for vehicle and IL-1β treated animals. Data expressed as mean ±SD.
Supplementary table II.

**Table II.** Physiologic parameters of vehicle, IL-1β, vehicle+BQ-123 and IL-1β+BQ-123 measured from 1 to 4h reperfusion

<table>
<thead>
<tr>
<th>Physiologic data</th>
<th>0 Hour</th>
<th>1 Hour</th>
<th>2 Hour</th>
<th>3 Hour</th>
<th>4 Hour</th>
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<tbody>
<tr>
<td>MAPB (mm Hg)</td>
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<tr>
<td>Vehicle</td>
<td>104±14</td>
<td>97±7</td>
<td>95±8</td>
<td>93±6</td>
<td>92±6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>94±14</td>
<td>85±6</td>
<td>82±8</td>
<td>82±10</td>
<td>85±4</td>
</tr>
<tr>
<td>Vehicle + BQ-123</td>
<td>96±15</td>
<td>93±9</td>
<td>99±9</td>
<td>97±8</td>
<td>94±7</td>
</tr>
<tr>
<td>IL-1β + BQ-123</td>
<td>103±13</td>
<td>100±6</td>
<td>104±6</td>
<td>103±6</td>
<td>101±6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Heart rate (bpm)</th>
<th></th>
<th></th>
<th></th>
<th>Average</th>
<th></th>
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<tr>
<td>Vehicle</td>
<td>365</td>
<td>334</td>
<td>376</td>
<td>358</td>
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<tr>
<td>IL-1β</td>
<td>353</td>
<td>399</td>
<td>379</td>
<td>377</td>
<td></td>
</tr>
<tr>
<td>Vehicle + BQ-123</td>
<td>410</td>
<td>401</td>
<td>374</td>
<td>395</td>
<td></td>
</tr>
<tr>
<td>IL-1β + BQ-123</td>
<td>301</td>
<td>312</td>
<td>352</td>
<td>322</td>
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</tr>
</tbody>
</table>

**Supplementary Table II:** Physiological parameters were monitored throughout the surgical and imaging procedures. Time course changes of temperature; MABP - mean arterial blood pressure and HR - heart rate, following intraperitoneal injection of vehicle, IL-1β and intravenous injection of BQ-123 following 60min MCAo (n=6-7) were measured. Temperature was maintained at 37 ±1°C for all animals. Data expressed as mean ±SD.
Supplementary figure I. Vasoactive mediator expression was studied in sham and MCAo animals treated with either vehicle or IL-1β. Brains collected 15 min after reperfusion were dissected, ipsilateral cortex and striatum removed, homogenized and qPCR performed. mRNA expression of COX-2 (A,B) and iNOS (C,D) were quantified and are shown as fold change from control (sham animals treated with vehicle) (One-way ANOVA, Dunn’s multiple comparison test) Data are presented as mean ± SD. (A-D n=5-6). ***P<0.001, *P<0.05.
Supplementary figure II. Endothelin receptor A (ETrA) (red) and alpha-smooth muscle actin (α-SMA) (green) expression was monitored in MCAo animals treated with either vehicle or IL-1β. ETrA was co-localized to α-SMA at 15min reperfusion in cortical arteries of MCAo animals receiving IL-1β unlike vehicle treated animals (arrowhead). Scale bar = 50µm.
Supplemental references


