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

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Abstract

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 (CBF) during reperfusion following experimental cerebral ischemia.

Methods—Serial diffusion and perfusion-weighted magnetic resonance imaging was performed following reperfusion in Wistar rats given systemic (intraperitoneal) interleukin-1 β (IL-1) or vehicle prior to 60min transient middle cerebral artery occlusion. The expression and location of endothelin-1 (ET-1) was assessed by PCR, ELISA and immunofluorescence.

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

Conclusions—These data suggests patients with a raised inflammatory profile may have persistent deficits in perfusion after re-opening 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.

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Disclosure

None.

Keywords

inflammation; cerebral blood flow; cerebral ischemia; magnetic resonance imaging; interleukin-1

Introduction

A raised systemic inflammatory profile is observed in co-morbidities associated with vascular disease, including hypertension, infection, atherosclerosis, obesity and diabetes¹⁻³. Observational clinical studies show that inflammation is independently associated with an increased risk of stroke as well as poor post-stroke outcomes^{4,5}. Robust preclinical evidence shows that systemic inflammation markedly exacerbates acute brain injury^{6,7}, but the underlying mechanisms remain unclear. The pro-inflammatory 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 ischemia⁸⁻¹⁰.

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 achieved¹¹. Recanalization is an established reperfusion therapy for acute stroke¹² however human imaging studies have shown that when recanalization is achieved it does not necessarily lead to restoration of tissue blood flow¹³. 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 recanalization¹⁴.

Pre-existing systemic inflammation may influence mechanisms needed to sustain adequate perfusion following 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 following cerebral ischemia. We show that systemic IL-1 markedly increases the extent of tissue hypoperfusion following 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 Data Supplemental section.

Animals

All experiments were performed using male, 9-week old, Wistar rats (350-450g) (Charles River Laboratories, UK) and adhered to the Animals (Scientific Procedures) Act, UK (1986).

Focal cerebral ischemia

Focal cerebral ischemia was induced by transient (60min) middle cerebral artery occlusion (tMCAo) using a modification of the intraluminal filament model originally described by Longa et al¹⁶. Physiological parameters were recorded and homeostasis maintained. Mortality rate was <15% in all groups except where stated. Rats were transcardially-perfused with 0.9% saline followed by 4% paraformaldehyde or 0.1% Diethyl Pyrocarbonate (DEPC) treated sterile saline at the conclusion of experiments.

Treatment

Treatment allocations were randomized and blinded until completion of analysis. Rat recombinant IL-1 β (4 μ g/kg; National Institute for Biological Standards and Controls, UK) 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 1mg/kg of the selective ET-1 receptor A (ETrA) antagonist BQ-123 (Merck Chemicals Ltd, UK) was administered at occlusion and reperfusion. BQ-123 was chosen to ablate the activation of ETrA due to its potent and specific inhibition of ET-1 contractility in rat vascular smooth muscle¹⁷.

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 described¹⁸.

MRI

Magnetic resonance imaging (MRI) was performed using a 7T horizontal bore magnet (Agilent, UK). Magnetic resonance angiography was used to assess occlusion and re-opening of the MCA. Lesion volume was determined from apparent diffusion coefficient (ADC) maps. Perfusion weighted imaging (PWI) was performed using pseudo-continuous arterial spin labeling^{19,20}.

MRI Analysis

Quantitative ADC maps were generated in Paravision 5 software (Bruker, Germany) from diffusion weighted images (DWI). 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 tissue²². Physiological measurements were analyzed using MATLAB (MathWorks Inc, USA).

Histological analysis

The volume of ischemic damage and hemorrhagic transformation was measured by staining coronal sections (360 μ m interval) with cresyl violet (Sigma, UK) and hematoxylin & eosin (H&E).

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 manufacturer's instructions. Detailed methods for the qPCR are described elsewhere²³.

Enzyme-linked immunosorbant assay

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

Immunofluorescence

Free floating coronal brain slices (30µm) were processed after tMCAo and immunofluorescence, microscopy and image analysis was performed as described in online data supplement.

Statistics

Group sizes (n=6-8) were calculated based on previous data. Data are presented as mean $\pm$ standard deviation (SD). Student's *t*-test was used for single comparisons and one-way or two-way analysis of variance (ANOVA) was followed by Bonferroni's correction for multiple comparisons or Kruskal-Wallis test followed by Dunn's test for multiple comparisons using Prism Graph 6.0 software (GraphPad Software, USA). 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-1 β , resulted in larger infarct volumes at 24h (vehicle: $66 \pm 19 \text{ mm}^3$; IL-1 β : $166 \pm 32 \text{ mm}^3$; $*P < 0.05$) (Fig. 1A). IL-1 β treated animals had a 3-fold higher volume of hypoperfused tissue 15min after re-opening the MCA, versus vehicle (vehicle: $22 \pm 7 \text{ mm}^3$; IL-1 β : $70 \pm 30 \text{ mm}^3$; $***P < 0.001$) (Fig. 1B). The volume of hypoperfused tissue in IL-1 β treated animals remained significantly higher up to 2h after MCA re-opening. Treatment with IL-1 β did not alter CBF in sham treated animals (data not shown). ADC-derived lesion volumes were significantly higher (2-fold) in IL-1 β treated compared to vehicle treated rats at 3 and 4h reperfusion ($**P < 0.01$) (Fig. 1C).

Upregulation of endothelin-1 mRNA and protein was associated with larger perfusion deficits in animals with systemic inflammatory challenge

Of the vasoactive mediators studied (supplementary Fig. I), a 2-fold increase in endothelin-1 (ET-1) mRNA expression was observed in the ipsilateral striatum ($**P < 0.01$) and cortex ($*P < 0.05$) of stroke+IL-1 β versus sham and vehicle treated counterparts (Fig. 2A&B). A 2-fold increase ($*P < 0.05$) in protein expression of ET-1 was measured in the cortex of IL-1 β treated MCAo animals (Fig 2C&D).

ET-1 receptor A is localized to smooth muscle in areas of perfusion deficits

Phosphorylated serine¹⁹ on myosin light chains (pMLC) (an integral step in brain endothelial cytoskeletal reorganization) and alpha smooth muscle actin (α -SMA) were co-localized in cortical arteries of IL-1 β treated animals at 15min and 4h after cerebral ischemia alone (arrowhead Fig. 3A).

ET-1 (closed arrowhead Fig. 3B) was identified in animals receiving IL-1 β prior to cerebral ischemia at both 15min and 4h after reperfusion, but in vehicle treated animals there was no ET-1 staining at 15min and minimal ET-1 staining at 4h. Vessels that stained positive for ET-1 also expressed endothelin-1 receptor A (ETrA) and were localized to the ipsilateral hemisphere of IL-1 β treated animals (supplementary Fig. 2).

Inhibition of ET-1 receptor A prevented IL-1 β induced hypoperfusion

A DWI-PWI mismatch was seen 15min, 2h, and 4h after MCAo in animals receiving vehicle, IL-1 β , vehicle+BQ-123 or IL-1 β +BQ-123 (Fig. 4A). Animals treated with IL-1 β had a significantly larger perfusion deficit at 15min (vehicle: $33 \pm 11 \text{ mm}^3$; IL-1 β : $73 \pm 26 \text{ mm}^3$; *** $P < 0.001$). In contrast, in animals treated with IL-1 β +BQ-123, the volume of hypoperfusion was reduced to levels seen in the vehicle treated group (IL-1 β : $73 \pm 26 \text{ mm}^3$; IL-1 β +BQ-123: $38 \pm 15 \text{ mm}^3$; *** $P < 0.001$) (Fig. 4B). ADC derived lesion volume was larger in IL-1 β treated than in vehicle treated rats at 4h reperfusion (vehicle: $26 \pm 10 \text{ mm}^3$; IL-1 β : $60 \pm 34 \text{ mm}^3$; *** $P < 0.001$). However, in animals treated with IL-1 β +BQ-123, the volume of infarction was markedly reduced at 4h reperfusion (IL-1 β : $73 \pm 26 \text{ mm}^3$; IL-1 β +BQ-123: $38 \pm 15 \text{ mm}^3$; *** $P < 0.001$), such that volumes were not significantly different to vehicle treated animals (Fig. 4C).

Blockade of ET-1 receptor A reduced ischemic damage and improved behavioral outcomes

Systemic IL-1 β challenge prior to MCAo exacerbated the extent of ischemic brain injury (3-fold) and functional deficits at 48h reperfusion (Fig. 5A&B). Conversely, administration of BQ-123 in the presence of IL-1 β and MCAo markedly attenuated (4-fold) ischemic damage and behavioral deficits (Fig. 5C). The incidence of HT (Fig. 5D) was not significantly increased in animals receiving BQ-123 (numbers of animals presenting with HT: vehicle, n= 2/6; IL-1 β , n= 1/6; vehicle + BQ-123, n= 2/6; IL-1 β + BQ-123, n= 1/7).

Acute systemic inflammatory challenge lowered CBF and was associated with a prothrombotic state

At 4h reperfusion, platelet CD41 positive immunostaining was seen within activated vessels of animals treated with systemic IL-1 β (Fig. 6A). Presence of IL-1 β was associated with significant accumulation of platelets (vehicle: 11 ± 7 per mm^2 ; IL-1 β : 52 ± 15 per mm^2 ; ** $P < 0.01$) and formation of small aggregates (vehicle: 10 ± 8 per mm^2 ; IL-1 β : 44 ± 31 per mm^2 ; * $P < 0.05$) (Fig. 6B) in the cortex of IL-1 β treated animals at 4h reperfusion. Treatment with BQ-123 did not completely deplete the number of platelets adhering to the endothelium at 4h reperfusion, though a trend towards reduction in platelet numbers was seen compared to IL-1 β alone (IL-1 β : 124 ± 57 per mm^2 ; IL-1 β +BQ-123: 61 ± 37 per mm^2 ; $P = 0.068$) (Fig.

6C). Blockade of ET-1 significantly reduced platelets in vehicle+BQ-123 treated animals (* $P < 0.05$) and reduced platelet aggregates of $< 10 \mu\text{m}^2$ and $10\text{--}30 \mu\text{m}^2$ in vehicle+BQ-123 and IL-1 β +BQ-123 treated animals at 4h reperfusion in the cortex (* $P < 0.05$).

Discussion

We have shown that systemic IL-1 β 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 post-stroke outcome^{24,25}. Elevated IL-1 β is a central component of these pathologies as seen in chronic and acute inflammatory models^{2,26}, thus administration of systemic IL-1 β is a relevant model for many conditions that predispose to stroke. It is thought that much of the detrimental actions of IL-1 β on ischemic stroke are exerted peripherally as levels of IL-1 β are undetectable in the brain¹⁰. Furthermore, IL-1 β has been shown to affect the magnitude of the acute phase response which is a key indicator or peripheral inflammation. IL-1 β causes an increase in C-reactive protein and IL-6, and in both preclinical¹⁰ and clinical studies²⁷, raised levels of these markers are associated with poor prognosis. Peripherally administered lipopolysaccharide (LPS), which mimics aspects of infection, has also been used to represent acute inflammation prior to stroke and mice treated with LPS just prior to stroke show a marked increase in ischemic injury, an effect that is inhibited by administration of the IL-1 receptor antagonist (IL-1Ra)¹⁰. Our recent data show that preceding pneumonia infection in mice also worsens ischemic brain damage, an effect dependent again on IL-1 β as well as platelet-endothelial interactions²⁸. This suggests endogenous IL-1 β 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-1 β concentrations^{29,30}. Previous studies have reported either no effect on blood vessel dynamics³¹ or a rise in cerebral blood volume³² in response to IL-1 β , but in both studies IL-1 β was administered in the absence of injury and it is likely that IL-1 β acts differently in the presence of ischemia^{33,34}. 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 endothelium³⁵ and neighboring cells³⁶. Previous experimental studies have shown the potent vasoconstrictive effects of ET-1³⁷ through its actions on ETrA on smooth muscle cells, in contrast to the predominantly vasodilatory ETrB that is abundant on endothelium and neurons³⁸. Both ETrA and B are upregulated following injury³⁹ but only blockade of ETrA before injury prevents hypoperfusion⁴⁰. Whilst antagonism of ETrA restored CBF and reduced ischemic damage in animals with a pre-existing inflammatory challenge, infarct was not reduced in stroke+BQ-123 treated animals

as seen in previous studies⁴¹. It is possible this discrepancy was due to the negligible volume of penumbral tissue remaining (as indicated by the DWI-PWI mismatch) for salvage by BQ-123. Furthermore, the observed increase in ET-1 expression and vessel contractility occurred primarily in the cortex of IL-1 β treated animals. This co-localizes 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^{42,43} and clinical setting⁴⁴. In patients with multiple sclerosis, an increase in plasma ET-1 was noted alongside a corresponding reduction in CBF as measured by PWI, while post-mortem studies show an upregulation of ET-1 in the brain parenchyma in MS⁴⁵. The vasoconstrictive actions of endothelium-derived ET-1 on smooth muscle are well elucidated but here we report that the presence of systemic IL-1 β acts as a trigger for vessel contractility via ET-1 following cerebral ischemia. It is possible IL-1 β activates the endothelium to increase production of ET-1 as both ischemic stroke and IL-1 are potent activators of the cerebrovasculature⁴⁶ and endothelial cells are a source of ET-1⁴⁷. 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^{13,48}. 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 stroke patients.

In conclusion, we have demonstrated that systemic inflammation may worsen outcomes after cerebral ischemia by IL-1 β -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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The effects of systemic inflammation on cerebral blood flow

Systemic inflammation was induced by i.p. IL-1 β and effects on infarct DWI-PWI were assessed after tMCAo. (A) Representative ADC maps with hypoperfused volume (red) and infarct core (black) overlaid, highlighted the mismatch between infarct (DWI) and CBF deficit (PWI) at 15min, 2h and 4h after reperfusion in vehicle (top) and IL-1 β treated animals (bottom). Cresyl violet staining shows infarct at 24h in vehicle and IL-1 β treated rats. Acute evolution of perfusion deficit (B) and ADC derived ischemic lesion volume (C) in vehicle and IL-1 β treated animals following 60min MCAo (two-way repeated measures ANOVA, Bonferroni post test). Data are presented as mean $\pm$ SD (n=8) *** P <0.001, ** P <0.01, * P <0.05.

Figure 2. Expression of early vasoactive mediators

Vasoactive mediator expression was studied in animals receiving vehicle or IL-1 β . Brains collected 15min after reperfusion were dissected, homogenized and qPCR performed. mRNA expression of ET-1 (A, B) were quantified and are shown as fold change from control (sham animals treated with vehicle). Protein levels of ET-1 (C, D) were measured in homogenized cortex and striatum at 15min reperfusion by ELISA. (One-way ANOVA, Dunn's multiple comparison test) Data are presented as mean $\pm$ SD. Scale bar = 50 μ m. (A-D n=6). ** P <0.01, * P <0.05.

Figure 3. The effects of systemic inflammation on vascular smooth muscle

Phosphorylated myosin light chain (pMLC) (red) immunoreactivity determined vasoconstriction in the ipsilateral hemisphere of MCAo animals at 15 min and 4h reperfusion in vehicle and IL-1 β treated animals. pMLC was co-localized to alpha-smooth muscle actin (α -SMA) (green) in cortical surface arteries of animals treated with IL-1 β only (arrowheads; **A**). Scale bar = 50 μ m. ET-1 expression was assessed in MCAo animals receiving vehicle or IL-1 β . Vehicle treated animals exhibited ICAM-positive blood vessels only (green) (open arrowheads; **B**) in striatum and cortex at 15min reperfusion. Conversely,

ET-1 (red) was observed in the vascular wall of IL-1 β treated animals at 15min and 4h reperfusion (arrowhead; **B**). Scale bar = 50 μ m.

Figure 4. BQ-123 reverses hypoperfusion induced by systemic inflammation

BQ-123 was used to antagonize ET-1 in rats receiving IL-1 β or vehicle. (A) Representative ADC maps with hypoperfused volume (red) and infarct core (black) overlaid demonstrating DWI-PWI mismatch at 15min, 2h and 4h after reperfusion in vehicle, IL-1 β , vehicle +BQ-123 and IL-1 β +BQ-123 treated animals. Volume of hypoperfusion (B) and ADC lesion volumes (C) were determined at each time point and compared between groups. (Two-way repeated-measures ANOVA, Bonferroni post test). Data are presented as mean $\pm$ SD (n=8).

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ in reference to stroke+IL-1 β versus stroke+vehicle, ###
 $P < 0.001$ in reference to stroke+IL-1 β +BQ-123 versus stroke+IL-1 β .

Figure 5. BQ-123 reverses IL-1 β -induced ischemic damage and improves neurological outcomes
Effects of BQ-123 on the extent of ischemic brain damage and behavioral and motor deficits were determined 48h after middle cerebral artery occlusion (MCAo). The volume of ischemic damage (**A**) (One-way ANOVA, Dunn's multiple comparison test) and the 28-point neuroscore (**B**) to assess sensorimotor deficits (two-way repeated measures ANOVA) was measured in animals receiving vehicle, IL-1 β , vehicle+BQ-123 and IL-1 β +BQ-123. Representative pictures from cresyl violet (**C**) and H&E stained tissue (**D**) 48h after MCAo illustrate differences in ischemic volume and hemorrhagic transformation (arrowheads).

Data are presented as mean $\pm$ SD (n=6-7). Scale bar = 2000 μ m. $**P<0.01$, $*P<0.05$. **Figure 5B:** $*P<0.05$ in reference to stroke+IL-1 β versus stroke+vehicle, $^{\#}P<0.05$ in reference to stroke+IL-1 β +BQ-123 versus stroke+IL-1 β .

Figure 6. Systemic inflammation is associated with hypoperfusion and a prothrombotic state
 The effect of systemic IL-1 β on platelet adhesion and aggregation was assessed after 60min ischemia. Immunofluorescence confirmed the presence of platelets in vessels of animals treated with IL-1 β at 4h (A). Total numbers of platelets and aggregates at 4h reperfusion were counted (B) (Student's t-test). Platelet numbers and aggregates were further analyzed in animals treated with vehicle+BQ-123 and IL-1 β +BQ-123 at 4h (C) (two-way ANOVA, Bonferroni's multiple-comparison post test). Scale bar = 50 μ m. Data expressed as average

number of cells/mm² in the predefined brain region and presented as mean $\pm$ SD. (n=5-6).

** $P < 0.01$, * $P < 0.05$.