Interleukin-1 receptor antagonist is beneficial after subarachnoid haemorrhage in rat by blocking haem-driven inflammatory pathology

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SUMMARY

Subarachnoid haemorrhage (SAH) is a major contributor to the burden of stroke on society. Treatment options are limited and animal models of SAH do not always mimic key pathophysiological hallmarks of the disease, thus hindering development of new therapeutics. Inflammation is strongly associated with brain injury after SAH in animals and patients, and inhibition of the pro-inflammatory cytokine interleukin-1 (IL-1) represents a possible therapeutic target. Here we report that a rupture of the middle cerebral artery in the rat produces heterogeneous infa rct patterns similar to those observed in human SAH. Administration of the IL-1 receptor antagonist (IL-1Ra) reduced blood-brain barrier breakdown, and the extent of breakdown correlated with brain injury. After SAH, haem oxygenase-1 (HO-1) was strongly expressed around the bleed site and in the cortex and striatum, indicating the presence of free haem, a breakdown product of haemoglobin. HO-1 expression was also found in the same regions as microglial/macrophage expression of IL-1α. The direct effect of haem on IL-1α expression was confirmed in vitro using organotypic slice culture (OSC). Haem-induced cell death was dependent on IL-1 signalling, with IL-1Ra completely blocking cellular injury. Furthermore, stimulation of mouse primary mixed glial cells with haem induced the release of IL-1α, but not IL-1β. Thus, we suggest that haem, released from lysed red blood cells (RBCs) in the subarachnoid space, acts as a danger-associated molecular pattern (DAMP) driving IL-1-dependent inflammation. These data provide new insights into inflammation after SAH-induced brain injury and suggest IL-1Ra as a candidate therapeutic for the disease.

INTRODUCTION

Subarachnoid haemorrhage (SAH) is an acute injury to the brain with devastating consequences. Its occurrence at a comparatively young age (compared with ischaemic stroke), poor outcome and high mortality rate mean that SAH is a major burden on society (Feigin et al., 2003; Feigin et al., 2005; Nieuwkamp et al., 2009). Inflammation is known to contribute to the worsening of acute brain injuries and to chronic brain diseases (e.g. Alzheimer’s disease) and might thus represent a therapeutic target (del Zoppo, 2010; Denes et al., 2010; Jin et al., 2010; Wyss-Coray, 2006). Indeed, inflammation is strongly associated with brain injury after SAH: increases in leukocytes, platelets and serum C-reactive protein (CRP) levels correlate with poorer outcome (Frijns et al., 2006; Denes et al., 2010). Furthermore, pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα), interleukin-8 (IL-8), high mobility group box-1 (HMGB-1) and IL-6 are elevated in cerebral spinal fluid (CSF) of patients with a worse clinical grade (Fassbender et al., 2001; Nakahara et al., 2009; Schoo et al., 2007).

The pro-inflammatory cytokine IL-1 is a key mediator of neuronal injury after acute brain injury (Allan et al., 2005), and inhibition of IL-1 actions represents a viable therapeutic strategy (Brough et al., 2011). The two most well characterised pro-inflammatory members of the IL-1 family are IL-1α and IL-1β, and production of these by macrophages and microglia is induced by activation of pattern recognition receptors (PRRs) such as those of the Toll-like receptor (TLR) family (Netea et al., 2008). During infection these PRRs are activated by pathogen-associated molecular patterns (PAMPs). It is not yet clear how they are activated during sterile inflammation (in the absence of infection), but endogenous molecules that are modified during disease or released by dead cells, known as danger-associated molecular patterns (DAMPs), might also activate PRRs (Chen and Nuñez, 2010).

SAH is considered a sterile injury and therefore might elicit a DAMP-mediated inflammatory response. The inflammatory response to sterile injury is similar to microbial infection, and the host receptors that mediate both could be the same (Chen and Nuñez, 2010). However, recent literature highlights sterile-specific mechanisms in the response to injury (Chen and Nuñez, 2010; Rock et al., 2010). Efforts are now being made to elucidate the specific DAMPs that elicit the inflammatory response in disease. Uric acid, released from dying cells, and its derivative, monosodium urate crystals, which cause the inflammatory disease gout, have been identified as molecules that initiate the sterile inflammatory response (Chen et al., 2006; Kono et al., 2010a). Furthermore, crystalline cholesterol found in atherosclerotic plaques, and serum amyloid A, an acute phase response protein in mice, have been recognised as DAMPs that exacerbate inflammation (Duewell et al., 2010; Niemi et al., 2011). The injection of necrotic cells into...
the peritoneal cavity of mice elicits an inflammatory response that is dependent on IL-1α and IL-1R1 (Chen et al., 2007).

Both IL-1α and IL-1β act at the type I IL-1 receptor (IL-1R1), initiating signalling cascades that result in the expression of inflammatory genes (Korherr et al., 1997; Smith et al., 2009). The endogenous IL-1 receptor antagonist (IL-1Ra) completely blocks signalling at the receptor, inhibiting inflammatory effects of IL-1α and IL-1β (Hannum et al., 1990). IL-1Ra is currently licensed as a therapy for rheumatoid arthritis (as Anakinra) and is the standard treatment for autoinflammatory diseases (Dinarello, 2011). IL-1Ra is also showing promise as an anti-inflammatory for the treatment of central nervous system (CNS) disease: a Phase 2 trial in acute stroke patients showed a reduction in inflammatory markers and improved clinical outcome at 3 months in patients receiving IL-1Ra compared with placebo control (Emsley et al., 2005).

The aim of this study was to investigate the role of IL-1 in brain inflammation in response to experimental SAH, to identify possible DAMPs as drivers of IL-1 expression and release, and test whether inhibiting endogenous IL-1 with recombinant IL-1Ra could improve outcome. We report that IL-1Ra, administered after SAH, was beneficial by reducing blood-brain barrier (BBB) breakdown, and the extent of this breakdown correlated with neuronal damage. Microglia were the major source of IL-1 following SAH. IL-1α expression was dominant compared with that of IL-1β, and was expressed as early as 12 hours after the insult. Haem oxygenase-1 (HO-1) expression after SAH suggested the presence of free haem throughout the brain. Furthermore, haem induced the release of IL-1α in primed mixed glial cells, enhanced IL-1α expression in organotypic slices culture (OSC) and increased cell death, an effect that was blocked by IL-Ra. Thus, we propose that haem, a breakdown product of red blood cells (RBCs), contributes to IL-1α production after SAH and that IL-1Ra could be beneficial in improving patient outcome.

RESULTS

IL-1α is produced by microglia/macrophages early after experimental SAH

To assess the role that IL-1 played in driving acute brain injury after SAH we initially investigated whether it was produced after SAH in the rat. Animals underwent a modified version of the intraluminal filament model of SAH (Park et al., 2008) and were sacrificed 12 hours after haemorrhage. Sham animals underwent identical surgery without perforation of the cerebral artery. Immunofluorescence on coronal sections from injured brains revealed that SAH induced the expression of IL-1α throughout the brain, including in the cortex and striatum (Fig. 1), in the hippocampus and especially in basal structures adjacent to the site of perforation (Fig. 1), where 46% of microglia were found to express IL-1α. Images shown are representative of immunofluorescent-positive cells seen in all animals 12 hours after SAH. IL-1β was also observed but to a much lesser extent and was restricted to a few cells in the striatum. IL-1α was expressed primarily by microglial/macrophage cells, because it colocalised with the ionised calcium binding adaptor molecule 1 (Iba1), which is expressed exclusively by microglia/macrophages (Ito et al., 1998). Microglia dynamically survey their environment (Nimmerjahn et al., 2005), but become activated in response to damage, changing their morphology from long, ramified protrusions to shorter, hyper-ramified branches (Streit et al., 1999). Our results indicate that both resting and fully activated microglia express IL-1α, because expression was observed in cells of different morphology (Fig. 1).

HO-1 is expressed throughout the brain early after SAH, in regions of IL-1α expression

During SAH there is an extravasation of RBCs that subsequently lyse, releasing haemoglobin (Hgb) and its oxidation product, haem. Haem is converted in brain by haem oxygenase (HO) into carbon monoxide, biliverdin and iron (Ascenzi et al., 2005). HO-1 is expressed primarily by glial cells and is inducible by RBC lysis products (Matz et al., 1997; Turner et al., 1998). HO-1 is expressed by glial cells and is inducible by RBC lysis products (Matz et al., 1997; Turner et al., 1998). Given that HO-1 knockout (KO) mice are more susceptible to sepsis-induced lethality, with free haem contributing to tissue damage (Larsen et al., 2010), we hypothesised that haem might contribute to brain inflammation and damage after SAH. At 12 hours after SAH, HO-1 was upregulated in the cortex, striatum, adjacent to the bleed site (Fig. 2), throughout the meninges and in the choroid plexus, when compared with sham-treated animals, in which it was not detectable. HO-1 is thought to be expressed primarily by glial cells and is induced by haem (Wagner et al., 2003). Previous studies have shown that haem is present throughout the brain after SAH (Lee et al., 2010; Turner et al., 1998), and the upregulation of HO-1 has been suggested as a marker of the presence of haem, although heat shock proteins and a variety of oxidants are also known to induce its expression (Lee et al., 2010; Turner et al., 1998). At 12 hours...
after SAH, HO-1 is localised to areas of microglial activation and IL-1α expression, but does not colocalise to microglial cells (Fig. 2).

### BBB breakdown is correlated with brain damage and is reduced by IL-1Ra

Experimental studies of SAH in rodents generally do not report any substantial neuronal cell death. This is either because the intervention does not produce infarcts, or because this was not reported as an outcome measure (Lee et al., 2009; Park et al., 2008; Prunell et al., 2003). We found previously, using the modified perforation model, that large blood loads produced focal yet heterogeneous infarct patterns, as seen in human patients (Greenhalgh et al., 2012; Rabinstein et al., 2005). Furthermore, there is a positive correlation between blood load and neuronal damage, where animals with a blood load grade ≥7 incur neuronal loss (Greenhalgh et al., 2012). Variability in blood load is high in this model (Sugawara et al., 2008). Therefore, to investigate the role of inflammation on brain injury after SAH effectively, we decided a priori to only assess animals that had a blood load grade ≥7 (Fig. 3).

Mortality after SAH was not affected by treatment: 21% (4/18) of placebo- and 20% (4/19) of IL-1Ra-treated animals died before 48 hours. There were no mortalities in the sham-operated group. Animals were randomly assigned to treatment groups, but not included in the study if they did not survive the initial haemorrhage. As a result, ten placebo and nine IL-1Ra-assigned (but not treated) animals died within moments of the perforation and were therefore not included in the mortality data.

BBB breakdown leads to extravasation of blood-borne protein and cells (vasogenic oedema), which can exacerbate inflammation and contribute to cellular damage (Simard et al., 2007; Simard et al., 2008). To test whether the IL-1 expressed 12 hours after SAH contributed to brain injury, animals underwent sham or SAH surgery and received either IL-1Ra (75 mg/kg) or placebo subcutaneously 15 minutes after haemorrhage. The effect of IL-1Ra on BBB breakdown after SAH was measured at 48 hours by quantifying the infiltration of endogenous rat IgG, a molecule normally excluded from the brain parenchyma by an intact BBB. In animals that received IL-1Ra there was significantly reduced BBB breakdown compared with placebo (Fig. 4A), with no IgG infiltration in sham animals.

Because neuronal damage in this experimental model of SAH is heterogeneous in its location and size, affecting areas such as the hippocampus, striatum and cortex (individually or in combination), we devised a neuronal damage scoring system to evaluate damage across all areas. Using this score we found that BBB breakdown correlated strongly with neuronal loss ($r^2=0.612$) (Fig. 4B). Although the reduction in brain damage in the IL-1Ra group was not significant at the $P<0.05$ level ($P=0.059$) compared with placebo (Fig. 4C), this might be due to higher blood load grades in the IL-1Ra group (Fig. 3). These data suggest that IL-1 is a driver of BBB breakdown after SAH, and its expression correlates with worsening injury.

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**Fig. 2. Early after SAH, HO-1 is found throughout the brain localised to brain regions expressing IL-1α.** Panels show representative coronal brain sections of SAH animals 12 hours after bleed (n=5). Representative images from the area adjacent to the bleed site, the cortex and the striatum are shown. HO-1-positive fluorescent cells (green); IL-1α-positive fluorescent cells (red). Areas of HO-1 immunofluorescence correspond to areas of microglial activation and IL-1α expression seen in Fig. 1. In sham animals, HO-1-positive fluorescence was undetectable. Scale bars: 100 μm.

**Fig. 3. Blood load grades 48 hours after SAH.** (A) Photomicrographs showing examples of blood load grades 48 hours after SAH. (B) Blood load grades were highly variable between animals; as expected, there was no significant difference between groups that went on to receive IL-1Ra (n=15) or placebo (n=14). (C) Blood load grades ≥7 were eligible for analysis.
Endovascular perforation model of SAH does not induce inflammatory changes in the periphery or CSF

Peripheral inflammation can contribute to brain injury in ischaemic stroke, and animal models of focal ischaemia reflect this (Denes et al., 2010; McColl et al., 2009; Wang et al., 2007). Less is known about the role of peripheral inflammation in SAH. To test the hypothesis that SAH increases peripheral inflammation that in turn could contribute to brain injury, we measured the levels of inflammatory mediators in blood before, and then at 3, 24 and 48 hours after, experimental SAH. The cytokines IL-1α, IL-6, TNFα, IL-10, interferon-γ and CXCL1 were all measured and did not change at any time point, apart from CXCL1, which showed a transient increase at 3 hours (Fig. 5A). However, this increase was due to surgical intervention, because SAH did not induce changes above those of sham animals.

SAH did not induce any changes in IL-1α, IL-6, TNFα, IL-10 and interferon-γ in CSF 48 hours after sham surgery or experimental SAH with IL-1Ra or placebo. This might be due to the single time point sampled or the sensitivity of the assay. Together, these findings indicate that, in this model of SAH, IL-1-mediated brain injury acts at the parenchymal level with limited input from circulating factors.

Haem, a product of RBC lysis, acts as a DAMP to induce expression and release of IL-1α

Hgb and its oxidation product, haem (Ascenzi et al., 2005; Rother et al., 2005), are rapidly distributed throughout brain tissue after SAH in rat (Turner et al., 1998). We therefore tested the hypothesis that free haem can act as a DAMP and drive inflammation after SAH. We applied haemin (the oxidation product of haem) to mouse
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Fig. 5. Experimental SAH does not induce inflammatory changes in the periphery or CSF. Log transformed CXCL-1 concentrations in plasma (A) and IL-6 concentrations in CSF (B). Two-way ANOVA showed a significant effect of time after bleed in CXCL-1 plasma concentrations, with no difference between sham or SAH groups. One-way ANOVA showed there was no significant difference in CSF IL-6 concentrations between groups (n=7-10).

Clinical relevance of SAH animal models

Clincially relevant animal models of disease are essential for the development of therapeutics, and guidelines set out by the stroke research community aim to improve the translation from preclinical research to clinical trials (Fisher et al., 2009). The severity of SAH and the heterogeneous outcome after a bleed have resulted in the implementation of many different animal models of SAH. Rodents are widely used in these models, for a variety of reasons, and these are reviewed and compared elsewhere (Gules et al., 2002; Lee et al., 2009; Prunell et al., 2003; Prunell et al., 2005; Titova et al., 2009). Of these models, it is generally accepted that intraluminal perforation of the internal carotid bifurcation most closely mimics clinical SAH; however, a high mortality rate and variation in blood loads are limitations of its use (Lee et al., 2009). Therefore, in the present study we used a modified intraluminal perforation model first described by Park et al. that employs a hollow polytetrafluoroethylene (PTFE) tube concealing a tungsten wire for a cleaner puncture of the artery wall, which reduces mortality (Park et al., 2008). This model faithfully represents human SAH, in the respect that brain injury is diffuse and highly heterogeneous between animals. Although representative of the human disease, the large inter-animal variation makes it difficult to assess outcome via stimulation of the P2X7 receptor and was used as a positive control in all IL-1 release experiments. Western blot analysis of supernatants revealed that haeminduced the cleavage of pro-IL-1α (31 kDa) to its mature form (17 kDa) through a calpain-1-dependent mechanism: release of mature IL-1α was blocked by the calpain inhibitor MDL28170 (100 μM) (Fig. 7). These results reveal haem as a potential DAMP in OSCs and mixed glial cells in vitro, and as a specific regulator of IL-1α expression and release. Recently, IL-1α has emerged as a key inflammatory mediator of sterile inflammatory responses (Chen et al., 2007; Kono et al., 2010b; Rock et al., 2010). This is the first report, to our knowledge, to show that haem can act as a DAMP in brain-derived cells, and is the first report of a DAMP that is selective for IL-1α over IL-1β.

DISCUSSION

Despite an aging population, mortality rates of SAH patients have declined over the past three decades owing to improved diagnostics and surgical interventions (Nieuwkamp et al., 2009). However, pharmacological treatment options for SAH are limited. The only licensed drug to treat SAH patients is nimodipine, a calcium channel antagonist, of which the benefit to patients is not universal (Dorhout Mees et al., 2008). Here, we report for the first time that IL-1Ra, an emerging CNS therapeutic, is beneficial in a clinically relevant animal model of SAH.

We have recently performed a dose-ranging study that shows that IL-1Ra given intravenously is safe in SAH patients and can achieve experimentally effective concentrations (for the treatment of ischaemic stroke) in the CNS, within a therapeutic time window (Galea et al., 2010). This study adds to the current promise of IL-1Ra as a neuroprotectant in stroke (Greenhalgh et al., 2011) and provides new evidence that brain injury after SAH could be reduced with IL-1Ra treatment. We are currently performing a double-blinded, randomised, placebo-controlled Phase 2 clinical trial investigating inflammatory markers in plasma and CSF after IL-1Ra treatment in SAH (N. Singh, N.J.R., S.M.A. et al., unpublished data) that will provide further information on the potential of IL-1Ra as a treatment for this disease.

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after SAH. To reduce variation between groups we only assessed animals with large blood loads for analysis, because these animals are more likely to have brain injury (Greenhalgh et al., 2012). Previous studies have often focussed on vasospasm, by measurement of basilar artery narrowing, as an outcome measure in animal models of SAH (Titova et al., 2009), despite there being little evidence that this correlates with neuronal cell loss in these models. Recent clinical evidence also shows that reduced vasospasm does not improve patient outcome (Vergouwen et al., 2011). Therefore, we believe that the current model represents a viable option for the investigation of neuroprotective drugs in SAH.

The mechanisms underlying brain injury and poor clinical outcome after SAH are complex. A severe complication for survivors of the initial bleed is delayed cerebral ischaemia (DCI) as defined by cerebral infarction identified on computed tomography (CT) or magnetic resonance imaging (MRI), and clinical deterioration (Vergouwen et al., 2010). The cause of DCI is unknown, but vasospasm (Macdonald et al., 2007), microemboli (Vergouwen et al., 2008), spreading depression (Dreier et al., 2009) and inflammation (Chaichana et al., 2010) have all been proposed to play a role, and it is likely that they act in combination.

A recent proposal is that extravasated RBCs in the subarachnoid space are the underlying driver of vasospasm and/or DCI. These RBCs are phagocytosed by infiltrating macrophages and neutrophils, and are then trapped, die and degranulate, releasing endothelins and oxygen free radicals, which result in inflammatory-induced arterial vasoconstriction (Chaichana et al., 2010). We hypothesised that the extravasated RBCs also provide haem, which acts as a DAMP to microglia and macrophages and exacerbates the inflammatory response via the production of IL-1α, worsening brain injury. We show that expression of HO-1 is upregulated throughout the brain after SAH and that IL-1α, but not IL-1β, is produced by glial cells in vivo after SAH and in vitro after haem stimulation. This evidence correlates well with important studies on sterile inflammation in the periphery in mice; these studies identify macrophages as the key cell type sensing sterile injury, and provide evidence that they produce IL-1α to elicit the inflammatory response (Chen et al., 2007; Kono et al., 2010b; Rock et al., 2010). The present study is the first to suggest that microglia/macrophages in the CNS sense sterile inflammation through the same IL-1α-mediated mechanism. It is also the first to identify haem as a DAMP that contributes to IL-1-mediated inflammation in SAH. Furthermore, we have recently shown that IL-1α expression is localised to areas of focal neuronal loss and penumbral tissues after experimental stroke (Luheshi et al., 2011). The present study provides further evidence that IL-1α is involved in cellular injury after a brain insult, because haem induced IL-1α expression and cell death in OSCs, an effect that was blocked with IL-1Ra treatment, and inhibiting IL-1α with IL-1Ra reduced measures of brain injury after in vivo SAH.

The release of haem after SAH is a result of RBC lysis in the subarachnoid space. The current work highlights the inflammatory actions of haem before it is metabolised by the HO enzymes to carbon monoxide, biliverdin and iron (Ascenzi et al., 2005), and other work describes the toxicity of haem independent of its iron metabolite (Dang et al., 2011). However, haem metabolism and the release of iron are also implicated in the pathogenesis of SAH (Borsody et al., 2006; Chaichana et al., 2010; Lee et al., 2010). Hgb released from RBCs is highly toxic but is scavenged by macrophages after it has been bound by the serum protein haptoglobin (Ascenzi et al., 2005). Humans express only one of three types of haptoglobin (α1-α1, α1-α2 or α2-α2) and the α2 subunit is associated with a lower Hgb binding affinity. SAH patients expressing the α2 subunit are more likely to suffer from vasospasm than those expressing α1-α1.
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In summary, IL-1Ra improves measures of brain injury in a clinically relevant model of SAH by inhibiting IL-1α-mediated sterile inflammation. We report haem as a newly identified inducer of IL-1α-mediated sterile inflammation after SAH, implicating it as a DAMP. The products of RBC lysis are abundant in the SAH brain and represent a major target for therapeutic intervention. This is the first report to highlight IL-1Ra as a possible treatment option for SAH and ongoing clinical trials will investigate this further.

METHODS

Animals

Studies were conducted on male Wistar rats (Charles River, UK) weighing 350-500 g under the UK Animals (Scientific Procedures) Act 1986. The animals were kept under a 12-hour light-dark cycle with free access to food and water.

Surgical procedures

Anaesthesia was induced (3%) and maintained (2%) by inhalation of isoflurane, 70% N2O and 30% O2, and core body temperature was maintained throughout the procedure at 37.0±0.5°C. For cerebral blood flow (CBF) monitoring using Laser Doppler (Oxford Optronix, UK), a midline scalp incision was made revealing the skull and bregma. A small burr hole was drilled to the last compact bone layer (5 mm lateral and 1 mm posterior to bregma). A drop of mineral oil was placed in the cavity to aid optical connectivity and the glass optical probe was placed into the cavity and fixed using Loctite superglue (Henkel, UK). Animals were then rotated to the supine position in preparation for the induction of SAH.

SAH

Experimental SAH was induced using a modification of the endovascular perforation model described previously (Park et al., 2008) and further characterised by our research group (Greenhalgh et al., 2012). Briefly, animals were laid supine and a midline neck incision was made, followed by blunt dissection and retraction of superficial tissue and muscle. The carotid arteries were visualised and the external carotid artery (ECA) was coagulated, cut and reflected inferiorly. A hollow PTFE tube (Braintree Scientific, SUBL-120; I.D.: 150 μm; O.D.: 300 μm), concealing a tungsten wire [75 μm diameter (Advent, UK)], was inserted into the right ECA and advanced ~18 mm along the internal carotid artery (ICA) until it reached the middle cerebral artery (MCA)-anterior cerebral artery (ACA) bifurcation, as verified by a drop in Laser Doppler signal. The tungsten wire was then advanced through the tubing to protrude 2 mm, perforating the MCA-ACA bifurcation; the wire and tube then were withdrawn immediately. A drop in blood flow that was maintained after withdrawal indicated successful perforation of the artery. Animals that did not survive the first 10 minutes after haemorrhage were not included in the study.

haptoglobin α1-α1 (Borsody et al., 2006). Oxidation of unscauged Hgb releases prosthetic haem groups, which we propose act as DAMPs to exacerbate inflammation and brain injury. Expression of HO-1 and/or the iron handling proteins transferrin (Tf), Tf receptor and ferritin is associated with experimental SAH brain injury, and iron chelation with deferoxamine (DFX) reduces injury (Lee et al., 2010). Furthermore, HO-1 increases brain injury after intracerebral haemorrhage, and bilirubin (a product of haem breakdown) and its oxidation products, known as BOXes, have been implicated in SAH pathogenesis (Clark and Sharp, 2006; Isikay et al., 2011; Wang and Dore, 2007). Thus, a picture is now emerging that several stages of RBC lysis (including activation of cellular processes that are involved in managing its products) might contribute to brain injury after SAH. As such, combinatorial approaches to target each step of this process could prove beneficial after SAH. DFX is safe in patients and is licensed to treat iron toxicity in haemochromatosis (Kontoghiorghes et al., 2005). In SAH, DFX is proposed to target the toxic end product of RBC lysis, iron, and could act synergistically with IL-1Ra, which we propose targets the inflammatory effects of haem. Thus, a dual therapy combing two clinically approved drugs could show increased efficacy and is an attractive option for future study.

Fig. 7. Haemin induces the release of IL-1α but not IL-1β in primary mixed glia. Graphs show the release of IL-1α and IL-1β in LPS-treated mouse primary mixed glial cells after haemin (30 μM; black bars) or vehicle (DMSO; white bars) for 1, 2, 4 and 6 hours. ATP (5 μM) stimulation for 1 hour was used as a positive control (striped bar). IL-1α release was significantly increased after 2, 4 and 6 hours of haem stimulation compared with vehicle (A). Haem stimulation had no effect on release of IL-1β (B). n=4, one-way ANOVA followed by Student’s t-test with Bonferroni’s correction; *P<0.05, **P<0.01, ***P<0.001. Western blot analysis revealed that haemin (30 μM) induced cleavage of pro-IL-1α (31 kDa) to the mature form of IL-1α (17 kDa) through a calpain-1-dependent mechanism, because release of mature IL-1α was blocked by the calpain-1 inhibitor MDL28170 (100 μM) (C).
animals, the PTFE tube was advanced to MCA-ACA bifurcation and then withdrawn without perforation. Animals were then immediately allowed to recover.

**CSF sampling**

Rats were anaesthetised and secured in a stereotaxic frame (Stoelting Co., IL). A midline incision was made between the ears, ending approximately 2 cm caudally. The fascia was retracted and muscles bluntly dissected to reveal atlanto-occipital membrane. A 28 gauge needle attached to a 1 ml syringe was used to pierce the membrane and aspirate CSF.

**Experimental groups**

To assess acute brain inflammation after SAH, rats underwent SAH ($n=6$) or sham ($n=3$) surgery and were allowed to recover for 12 hours. To investigate the inflammatory response after experimental SAH, and in particular the role of IL-1, animals were randomised to three groups; sham ($n=9$), SAH receiving IL-1Ra ($n=19$) or SAH receiving placebo ($n=18$). SAH groups received a single subcutaneous (s.c.) dose of human-IL-1Ra (r-met-huIL-1RA: Kineret; Amgen, Thousand Oaks, CA) or placebo (Amgen), at 75 mg/kg, 15 minutes after SAH; experimenter was blinded to treatment group. Blood samples were taken from the tail vein immediately prior to surgery and 3 and 24 hours after SAH. At 48 hours, animals were re-anaesthetised and CSF and cardiac blood were sampled. Rats were perfused transcardially with 120 ml of saline solution (PFA; 4%). Brains were removed immediately post-fixation (4%) for 24 hours before cryoprotection in cold paraformaldehyde solution (PFA; 4%). Brains were cryoprotected and then photographed at high resolution (Qimaging, Canada). Images of the brains were then analysed using a recently characterised grading system for bleed load after SAH in rats (Sugawara et al., 2008). Briefly, the basal cistern was divided into six segments. Each segment was allotted a grade from 0 to 3 depending on the amount of subarachnoid blood clot in the segment as follows – Grade 0: no subarachnoid blood; Grade 1: minimal subarachnoid blood; Grade 2: moderate blood clot with recognisable arteries; Grade 3: blood clot obliterating all arteries within the segment. The animals received a total score ranging from 0 to 18 after adding the scores from all six segments. A previous study from our laboratory showed positive correlation between blood load and neuronal damage (Greenhalgh et al., 2012), and only animals with a blood grade ≥7 incur neuronal loss. Therefore, decided a priori to only assess animals that had a blood load grade ≥7.

**Histological and immunohistochemical analysis**

Coronal brain sections were cut 30 μm thick using a freezing slide microtome and stored free floating in cryoprotectant solution (6.80 g Na₂HPO₄ × 2H₂O, 0.79 g NaH₂PO₄ × H₂O, 500 ml dH₂O, 200 ml glycerol, 300 ml ethylene glycol). Brain sections were mounted and the Nissl stain cresyl violet (Sigma, UK) was used to assess cell morphology and infarct location. Brain sections were analysed under a light microscope and areas of neuronal cell death recorded.

Double immunofluorescence was used with the following antibodies: anti-IL-1α or anti-IL-1β raised in goat (1:100; R&D Systems, UK); microglial marker anti-Iba1, raised in rabbit (1:1000; Wako Chemicals, Germany); anti-HO-1 raised in rabbit (1:400; Enzo Life Sciences, UK). After blocking in 2% normal donkey serum (Vector Laboratories, CA) sections were incubated overnight at 4°C in primary antibodies, which were then visualised with the appropriate fluorochrome-conjugated secondary antisera (Alexa-Fluor-594 (1:500) or Alexa-Fluor-488 (1:500); Molecular Probes). Mounting medium Prolong Gold with DAPI (Invitrogen, UK) was used to mount sections. For immunohistochemistry, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide (Sigma, UK) in dH₂O and sections were treated with 2% normal serum (Vector Laboratories, CA) for 1 hour at room temperature. Biotinylated rabbit anti-rat IgG antibody (1:500; Vector Laboratories, UK) was visualised with the avidin-biotinylated peroxidase complex (Vectastain ABC elite kit) and its substrate diaminobenzidine (DAB; Vector Laboratories, UK). The same procedures were followed using tissue sections without adding primary antibodies and were used as controls for nonspecific secondary antibody labelling. Brightfield images were collected on an Axioam camera CCD camera (Zeiss, Germany) upright microscope through Axiovision software (Zeiss, Germany). Fluorescent images were collected on an Olympus BX51 upright microscope and captured using a CoolSNAP ES camera (Photometrics, UK) through MetaVue software (Molecular Devices, UK). Specific band-pass filter sets for DAPI, FITC and Texas Red were used to prevent bleed-through from one channel to the next. For quantification of IL-1α-positive microglial cells, total Iba1-positive cells were counted at 20× magnification, immediately adjacent to the site of perforation ($n=5$). The number of these cells that were also IL-1α positive was expressed as a percentage of the total number of microglial cells.

**Assessment of neuronal damage**

We devised a semi-quantitative measure of neuronal damage that assesses cell death across brain regions as follows: brains were divided into three regions (hippocampus, cortex and striatum) and scored using the following criteria: 0, no neuronal damage; 1, slight histological abnormalities; 2, focal areas of neuronal loss; and 3, majority of neurons in the region lost. Animals received a total score from all three regions.

**Assessment of BBB damage**

To assess BBB damage, after immunohistochemical staining for endogenous rat IgG, nine coronal sections were selected that neuroanatomically corresponded to the following coordinates in the rat atlas (Paxinos and Watson, 1998) with respect to bregma (4.2, 3.2, 1.2, −0.26, −1.3, −1.8, −3.14, −4.8, −6.8 mm). This was based on the initial report, which showed that eight sections were sufficient to accurately calculate volumes in the damaged hemisphere (Osborne et al., 1987). Because the region of IgG infiltration was almost exclusively in the ipsilateral hemisphere, the extent of BBB breakdown was corrected for oedema as described previously (Lin et al., 1993). Areas of BBB damage were determined and the total volume was quantified by creating a curve using GraphPad Prism of
immunopositive area against distance between coronal levels. The area under the curve represented the volume of BBB breakdown. Blinding to treatment groups was carried out during histological, immunofluorescent and immunohistochemical analysis.

**Plasma, cerebrospinal fluid and liver cytokine quantification**

The concentrations of IL-1α, IL-6, TNFα, IL-10 and IFNγ were quantified using cytometric bead array (CBA) Flex Sets (BD Biosciences) according to the manufacturer’s protocol in: plasma, prior to surgery and 3, 24 and 48 hours after SAH; and in CSF and liver 48 hours after SAH. CXCL1 was measured, at the above times, using an ELISA (enzyme-linked immunosorbent assay) DuoSet kit (R&D Systems, Abingdon, UK).

**OSCs**

For hippocampal OSC, brains of C57BL/6J mice (postnatal day 6-7) were prepared according to the protocol described previously (Stoppini et al., 1991). Briefly, 300-μm transverse sections were cut using a vibrating microtome (Leica Microsystems, UK). Hippocampi were dissected out and transferred to 0.4-μm porous membrane inserts (Millipore, Watford, UK). Four hippocampal sections were plated on each 30 mm insert in a six-well plate containing 1 ml of medium [50% HEPES buffered-MEM, 25% heat-inactivated horse serum, 24% HBSS with 2 mM glutamine and 1% penicillin/streptomycin (P/S)]. OSCs were maintained at 37°C, 5% CO₂. Full media change was made the next day and every other day until treatment. On day 6, OSCs were transferred to serum-free media containing either vehicle (DMSO) or haemin (30 μM) for 2, 4 or 6 hours with or without recombinant human IL-1Ra (100 or 500 ng/ml). LPS (1 μg/ml) was used as a positive control. For cell death analysis, PI was added to the media and the OSCs were returned to the incubator for 30 minutes. Afterwards, OSCs were washed with PBS and fixed with PFA 4% for 10 minutes, washed and mounted using Prolong Gold (Invitrogen, UK). PI intensity was analysed using ImageJ (NIH Image). For protein analysis, OSC lysates were prepared using PBS containing 0.1% Triton X-100 and protease inhibitor cocktail (Calbiochem, UK).

**Preparation of cell cultures**

Mixed glia were cultured from C57BL/6J mice at postnatal day 1-4 as previously described (Pinteaux et al., 2002). Briefly, cerebral cortices were stripped of their meninges and dissociated by trituration in DMEM containing 10% fetal bovine serum (FBS) and 1% P/S. Cells were maintained at 37°C, 5% CO₂. Full media change was made the next day and every other day until treatment. On day 6, OSCs were transferred to serum-free media containing either vehicle (DMSO) or haemin (30 μM) before the addition of haem. Untreated cultures were used as a negative control.

**ELISA**

Murine IL-1α and IL-1β were quantified by ELISA (DuoSet, R&D Systems, UK) according to the manufacturer’s instructions. Cell lysates were prepared by incubating cultured cells on ice for 10 minutes in 0.1% Triton in PBS with protease inhibitors (Calbiochem, UK).

**TRANSLATIONAL IMPACT**

**Clinical issue**

Subarachnoid haemorrhage (SAH), a form of stroke that can occur spontaneously or as a result of a head injury, is a major burden on society. The loss of productive life years from SAH is comparable to that of cerebral infarction, the most common form of stroke. Despite this, pharmacological treatment options for SAH are restricted to nimodipine, a calcium channel agonist with limited efficacy. Appropriate animal models of SAH mimicking human pathophysiology are essential for the development of neuroprotective drugs, but the neuronal loss observed in human SAH is generally not seen in experimental models.

**Results**

The authors used a recently described protocol to induce SAH in rats that causes widespread neuronal loss. They show that blocking the pro-inflammatory cytokine interleukin-1 (IL-1) using its endogenous receptor antagonist, IL-1Ra, provides benefits in this model by reducing blood-brain-barrier breakdown, a feature that is correlated with neuronal damage. In addition, they found that haem oxygenase-1 (HO-1) was expressed around the bleed site and in the cortex and striatum, indicating the presence of free haem, a breakdown product of haemoglobin that is released from lysed red blood cells in the subarachnoid space. A direct link between haem-induced cell death and IL-1 was confirmed using organotypic slice culture, which showed that haem-induced neuronal death was dependent on IL-1, and that cells predominantly expressed the IL-1α isoform. These results suggest that haem acts as a danger-associated molecular pattern (DAMP) driving inflammation in SAH.

**Implications and future directions**

IL-1Ra is a licensed therapy for rheumatoid arthritis, and is the standard treatment for auto-inflammatory diseases. It is also being tested as treatment for stroke and other disorders. Using an animal model that is appropriate for translating novel therapeutics, this study provides the first preclinical evidence that IL-1Ra might also be beneficial in SAH. These new findings also outline a mechanism of action that implicates haem as a driver of central nervous system (CNS) inflammation. Notably, haem has recently been implicated as a key mediator of severe systemic inflammation in sepsis. This and the data presented here lead the authors to propose that free haem acts as a DAMP that initiates inflammation predominantly through the expression and release of IL-1α (as described for other sterile insults). Thus, IL-1α seems to be a ke key pro-inflammatory cytokine in acute brain injury after SAH, indicating that inhibition of the IL-1 pathway through blocking IL-1β alone might not be an effective treatment. Furthermore, IL-1α should now be considered a potential player in other forms of brain injury, including stroke. Ongoing studies are investigating CNS and peripheral inflammation in SAH patients being treated with IL-1Ra or placebo, and assessing the effects of treatment on outcome. Future work should consider the prophylactic use of IL-1Ra (in SAH patients with delayed cerebral ischaemia) and investigate targeting other products of the red blood cell lysis pathway in combination with IL-1Ra; for example, with the iron chelator deferoxamine, which is also beneficial in experimental SAH.

**Western blot**

Mixed glial supernatants were harvested and prepared in sample buffer containing 1% β-mercaptoethanol. Samples were boiled and then electrophoresed on 12% SDS-acrylamide gels. Proteins were transferred to a nitrocellulose membrane and blotted with a goat anti-mouse IL-1α polyclonal antibody (R&D Systems, UK) followed by a HRP-conjugated anti-goat antibody, and subsequently detected using an ECL detection system (Amersham, UK).
using enhanced chemiluminesence reagents (ECL, GE Healthcare, UK).

Statistical analysis
Data were analysed using Student’s t-test for comparing two groups; one-way ANOVAs followed by Student’s t-test with Bonferroni’s correction for comparing two or more groups; two-way ANOVAs followed by Student’s t-test with Bonferroni’s correction for experiments with two independent variables; and, for relationships, linear regression analysis. Data are expressed as the mean ± s.d. ***P<0.001, **P<0.01, *P<0.05.

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COMPETING INTERESTS
N.J.R. is a non-executive Director of AstraZeneca but no part of this work involved AstraZeneca.

AUTHOR CONTRIBUTIONS

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