Journal of Cerebral Blood Flow & Metabolism (2014) 34, 759–763
© 2014 ISCBFM All rights reserved 0271-678X/14 $32.00
www.jcbfm.com

BRIEF COMMUNICATION

Role of unphosphorylated transcription factor STAT3 in late cerebral ischemia after subarachnoid hemorrhage

Ajoy K Samraj[1], Anne H Müller[1], Anne-Sofie Grell[1] and Lars Edvinsson[1,2]

Molecular mechanisms behind increased cerebral vasospasm and local inflammation in late cerebral ischemia after subarachnoid hemorrhage (SAH) are poorly elucidated. Using system biology tools and experimental SAH models, we have identified signal transducer and activator of transcription 3 (STAT3) transcription factor as a possible major regulatory molecule. On the basis of the presence of transcription factor binding sequence in the promoters of differentially regulated genes (significant enrichment PE: 6×10^5) and the consistent expression of STAT3 (mRNA, $P = 0.0159$ and Protein, $P = 0.0467$), we hypothesize that unphosphorylated STAT3 may directly DNA bind and probably affect the genes that are involved in inflammation and late cerebral ischemia to influence the pathologic progression of SAH.

Journal of Cerebral Blood Flow & Metabolism (2014) **34,** 759–763; doi:10.1038/jcbfm.2014.15; published online 12 February 2014

Keywords: gene signatures; subarachnoid hemorrhage; JAK2-STAT3 signaling; transcription factor binding sites

INTRODUCTION

Subarachnoid hemorrhage (SAH) is often due to the rupture of an arterial aneurysm or a vascular malformation with severe and detrimental clinical outcome. Delayed cerebral ischemia that follows days after SAH is associated with pathologic vasoconstriction and exacerbated neurologic damage.[1] Understanding the molecular mechanisms that lead to late cerebral ischemia might provide novel pharmacological targets for the SAH therapeutics.

Studying gene signatures, a set of genes that are expressed at a certain physiologic or pathologic condition, may be very informative and reveal new aspects of particular biologic processes.[2] The present study was designed to use such large set of data for the identification of responsible regulatory factors, in particular transcription factors that are involved or responsible for the upregulation of cerebrovascular and inflammatory factors/genes. The first part of the study employs a combination of system biology tools for the identification of enriched transcription factor binding sites (TFBSs) of differentially expressed gene (DEG) sets in a microarray of cerebral arteries subjected to SAH. The second part visualizes how the identified transcription factor exerts control over the DEG sets using gene network analysis.

Here, we focus on signal transducer and activator of transcription 3 (STAT3), a pivotal part of JAK-STAT signaling cascade known to regulate several acute-phase protein genes.[3] Several cytokines and growth factors that are known to activate STAT3 have been reported in ischemic and hemorrhagic stroke.[4] However, its role in hemorrhagic stroke still needs to be established. In this study, using system biology tools and experimental SAH, we show that the accumulation of unphosphorylated STAT3 in the cerebral arteries might have a pivotal role in orchestrating the expression of genes that are involved in the late cerebral ischemia and related pathogenesis after SAH.

MATERIALS AND METHODS

Rat Subarachnoid Hemorrhage Model

All experiments were conducted in full compliance with the guidelines set forth in the European Council's Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes, and were approved by the Danish National Ethics Committee (Danish Animal Experimentation Inspectorate license no. 2011/561-2025). Induction of SAH was performed in 11 weeks old male Sprague-Dawley rats (300 to 350 g) as described previously.[5] To induce SAH, 300 μL of blood was withdrawn from the tail artery and injected intracisternally.

Transcription Factor Binding Site Enrichment Analysis

Differentially expressed genes having a *q* value of 0.05 were selected and subsequently analyzed by an up-to-date version of the transcription factor binding analysis program, SMART, as previously described.[6] The *q* values are statistical *P* values that have been optimized to reduce the false discovery rate in a multiple testing set-up. The false discovery rate is optimized by using characteristics of *P* value distribution to produce a list of *q* values. Briefly, the promoter regions of the DEGs were scanned for TFBSs. Two criteria for significance have been employed, significantly enriched TFBS in terms of total number of binding sites for the promoters, and significantly present TFBS in terms of the fraction of promoters with binding sites. Identification of significant TFBS was performed by a resampling procedure in which the query gene set is compared with typically 10^5 gene lists of similar size randomly drawn from the TFBS/promoter database.

Gene Network Analysis

Gene network analysis was carried out using the Ingenuity Pathway Analysis software (www.ingenuity.com). Ingenuity Pathways Knowledge Base was used as an information source to develop ingenuity pathway analysis gene network maps within global molecular network.

[1]Department of Clinical Experimental Research, Glostrup Research Institute, University of Copenhagen, Glostrup, Denmark and [2]Division of Experimental Vascular Research, Department of Clinical Sciences, Lund University, Lund, Sweden. Correspondence: Dr AK Samraj, Department of Clinical Experimental Research, Glostrup Research Institute, University of Copenhagen, Ndr. Ringvej 69, 2600 Glostrup, Denmark.
E-mail: aksamraj@gmail.com
Received 5 August 2013; accepted 8 January 2014; published online 12 February 2014

Quantitative Real-Time PCR

RNA extraction and cDNA synthesis were performed according to the kit manufacturer's protocols (Machery-Nagel, Düren, Germany and Qiagen, Hilden, Germany). Quantitative real-time PCR was performed using the SYBR Green kit (Qiagen) on a CFX384 Real-Time System (Bio-Rad, Hercules, CA, USA) and normalized to β-actin, GAPDH, and EF1-α. Rat-specific STAT3, interleukin 6 (IL-6), and Janus kinase 2 (JAK2) primers were obtained from SA Biosciences (Qiagen). For statistical significance, *t*-test was performed between sham and SAH groups and $P < 0.05$ was considered as significant.

Immunohistochemistry

Immunohistochemistry of middle cerebral artery was performed as described previously.[5] Middle cerebral artery sections were incubated with primary antibodies against STAT3 (Cell Signaling Technology, Beverly, MA, USA, #9139; 1:500) and IL-6 (Abcam, Cambridge, UK, #ab6672; 1:250). After the secondary antibody incubation, visualization was performed using an epifluorescence microscope (Nikon 80i, Tokyo, Japan). Staining with secondary antibodies alone was performed to establish that the staining was not due to the unspecific binding of secondary antibodies.

Western Blots

Western blot analysis of cerebral arteries was carried out as described previously.[5] The membranes were probed with phospho-STAT3 (T705) (Cell Signaling Technology, #9131), STAT3 (Cell Signaling Technology, #9139), IL-6 (Abcam, #ab6672), β-actin (Santa Cruz Biotechnology, SC-47778) and horse radish peroxidase-conjugated secondary antibody, respectively. Enhanced chemiluminescence reagents were used to visualize the protein. Band intensity was measured and *t*-test was performed between sham and SAH groups for statistical significance ($P < 0.05$).

ARRIVE Statement

The manuscript has been written in accordance with ARRIVE guidelines.

RESULTS

Transcription Factor Binding Site Enrichment and Gene Network Analysis of Differentially Expressed Genes after Subarachnoid Hemorrhage

Gene expression profiling is a widely applied method to understand the biologic state in response to an exogenous stimulus. Transcription factors have a significant role in orchestrating the gene signature to define such a biologic state. Therefore, a differentially regulated gene set from a microarray of cerebral arteries at 24 hours after SAH was analyzed for the identification of TFBS enrichment using the systematic motif analysis retrieval tool (SMART) software.[6] We found at least three significantly enriched transcription factors: STAT3 (significant enrichment (PE): 6×10^5), Ikaros family zinc finger 1 (IK1) (PE: 4×10^5), and the transcriptional regulator protein Bach2 (PE: 4.4×10^4). In addition to PE values, parameters such as the number of hits in the genomic library and the percentage of DNA binding motif present among the DEGs in the array were also taken into consideration (see Supplementary Table T1) to choose the right candidate for further validation. IK1 is primarily involved in the development of adaptive immune system and chromatin remodeling to control gene expression.[7] Until now, its role in stroke and inflammation is not clearly elucidated. Bach2 is a transcriptional regulator that acts by binding to musculoaponeurotic fibrosarcoma sequences.[8] Due to the low number of hits (21) and the low percentage of DNA binding motifs (20%) Bach2 was excluded from the study. In contrast to the IK1, STAT3 is shown to be regulated by genes of several acute-phase proteins that are shown to be activated upon hemorrhagic stroke.[9,10] Therefore, STAT3 was chosen for further validation. The complete list of enriched transcription factors is provided in Supplementary Table T1. To visualize the 'gene network in operation', DEGs from the microarray were subjected to gene network analysis using ingenuity pathway analyzer. It graphically represents the molecular relationships between genes, and biologic relationships using the data from well-established studies. Figure 1A represents the gene network pathway generated by the software (note: STAT3 was later introduced into the network). The network analysis indicated a possible indirect regulatory role for IL-6 over several DEGs. Interleukin 6 has been shown to be upregulated upon cerebral ischemia that is pivotal for the inflammation and tissue repair process.[11] Despite the significant enrichment for the binding sites, STAT3 was absent from the gene network pathway but when STAT3 was introduced into the network, it indicated a possible direct interaction/regulatory role over several DEGs, including IL-6. All directly interacting (uninterrupted line) genes are reported to contain STAT3 binding sites (Figure 1A).

Increased JAK2-STAT3 Transcription and Accumulation of Unphosphorylated STAT3 in Experimental Subarachnoid Hemorrhage

Since TFBS enrichment and gene network analysis indicated a plausible role for STAT3, the involvement of the JAK2-STAT3 signaling pathway in SAH was evaluated. Janus kinase 2 is a nonreceptor tyrosine kinase that is implicated in the signaling mediated by gp130-linked cytokines such as IL-6 *via* STAT3.[12] Therefore, we investigated the expression status of IL-6 (the ligand), JAK2, and STAT3 at 24 hours after SAH. No significant change in the mRNA of JAK2 or STAT3 was observed but the augmented expression of IL-6 mRNA and protein indicated that STAT3 still could actively be inducing transcription (Figures 1B to 1E). Such scenario is possible if the transcription/translation event has taken place at an early stage of SAH insult. Since the activation status of STAT3 is defined by the phosphorylation, the phosphorylation status of STAT3 (S707) was evaluated by western blot analysis. No pronounced increase in phospho-STAT3 levels was observed at 24 hours of SAH (Supplementary Figure S1). Interestingly, a significant increase in the expression of unphosphorylated STAT3 was observed with both western blot and immunohistochemistry (Figures 1E to 1G), which did not correlate with the unaltered mRNA levels. Such scenario with STAT3 has previously been evidenced in cancer and inflammation.[13,14]

Therefore investigation of an early time point, 6 hours after SAH, was carried out to establish the link between STAT3 mRNA and protein expression. A significant increase in the mRNA of JAK2, STAT3 and IL-6 was observed (Figures 2A to 2C). In correlation, a pronounced increase in the levels of unphophorylated-STAT3 protein as well as phosphorylated STAT3 (T705) was evidenced (Figures 2D to 2G). Taken together, the results of 6 and 24 hours of SAH indicate that the accumulation of unphosphorylated STAT3 in the cerebral arteries may influence the gene expression to affect the pathologic progression after SAH insult. To substantiate the notion, cerebral arteries from a group of SAH rats were immunohistochemically tested for the sustained STAT3 accumulation at later time points after SAH. As expected, we observed accumulation of STAT3 and IL-6 expression even at 48 hours after SAH (Supplementary Figure S2).

DISCUSSION

Delayed cerebral vasospasm followed by ischemia brings severe neurologic damage or death to two-thirds of the patients with severe SAH. A number of compounds have been tried without any clinical success.[15] Lessons from such studies indicate that identification of major regulatory candidates that would affect wide range of genes is vital to halt/influence the pathologic progression of SAH. The present study, to our knowledge, is the first attempt to deduce SAH gene signatures for the identification of possible major regulatory molecules involved in SAH pathophysiology. So far, all previously reported gene expression profiling studies were prematurely concluded by functionally grouping the DEGs.[16] Such conclusion did not help to deduce any possible major regulators.

© 2014 ISCBFM

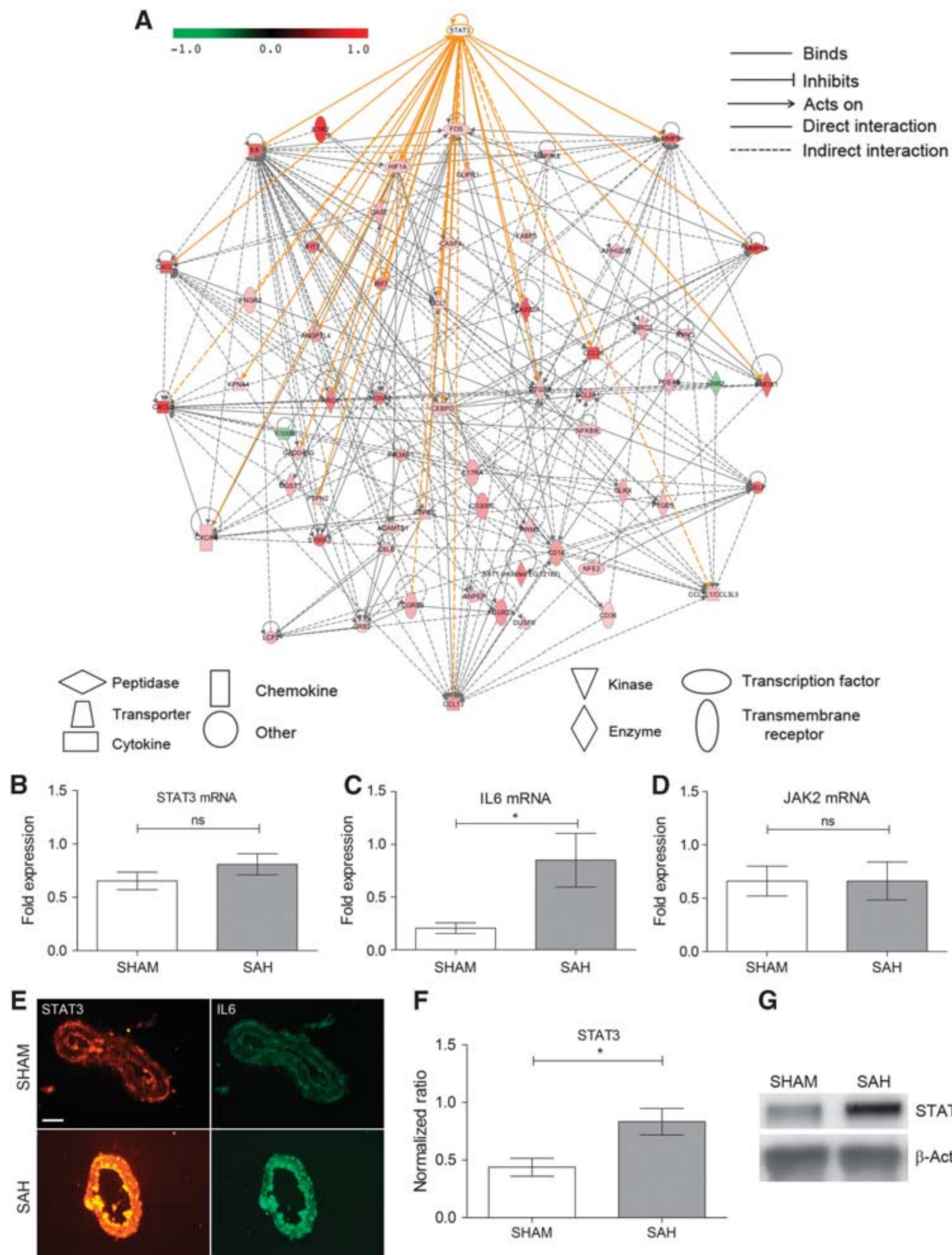

Figure 1. Gene network analysis of differentially expressed genes (DEGs) and validation after subarachnoid hemorrhage (SAH). (**A**) The network analysis was performed for the DEGs of SAH microarray (24 hours) using the Ingenuity Pathways Analysis software (Ingenuity Systems, www.ingenuity.com). For each gene, its corresponding gene object was mapped using Ingenuity Pathways Knowledge Base (IPKB) and overlaid onto a global molecular network developed from information contained in the IPKB. Signal transducer and activator of transcription 3 (STAT3) was introduced into the network to visualize its control over the network. The color coding represents the differential expression of genes which is adopted from the heat map generated after 24 hours of SAH. (**B**–**D**) Interleukin 6 (IL-6) ($P = 0.0477$), Janus kinase 2 (JAK2) ($P = 0.9985$), and STAT3 ($P = 0.2804$) mRNA expression levels at 24 hours of experimental SAH ($n = 4$, per group). (**E**) Representative immunohistochemical staining of STAT3 and IL-6 after 24 hours of SAH. Sections of middle cerebral arteries are shown as representative results of SHAM and SAH operated rats ($n = 4$, per group). The scale bar is 50 μm. (**F**) Quantification of immunoblots for STAT3 expression after 24 hours ($P = 0.0467$). Quantification presented as mean ± s.e.m. of ratio band intensity that is normalized to β-actin expression. ($n = 4$, per group). For statistical significance, *t*-test was performed between SHAM and SAH groups and $^*P < 0.05$ considered as significant. (**G**) Representative immunoblots showing the expression of STAT3 after 24 hours of SAH.

In this study, we have analyzed the promoters of the DEGs for TFBS enrichment from a microarray of cerebral arteries subjected to SAH. At least three transcription factors (STAT3, IK1, and Bach2) were identified as the most significantly enriched regulatory factors (Supplementary Table 1). Due to the low number of hits and the percentage of DNA binding domains, Bach2 was excluded from the study. Signal transducer and activator of transcription 3 was chosen over IK1 due to its association with acute-phase proteins and inflammatory cytokine regulation.[9,10] As substantiation to the finding, we noted a significant increase in the transcripts of IL-6 and JAK2, the essential components of STAT3 activation, in the cerebral arteries after experimental SAH. In agreement with the notion that the phosphorylation of STAT3 is essential for its activation, we noted a significant increase in the

© 2014 ISCBFM

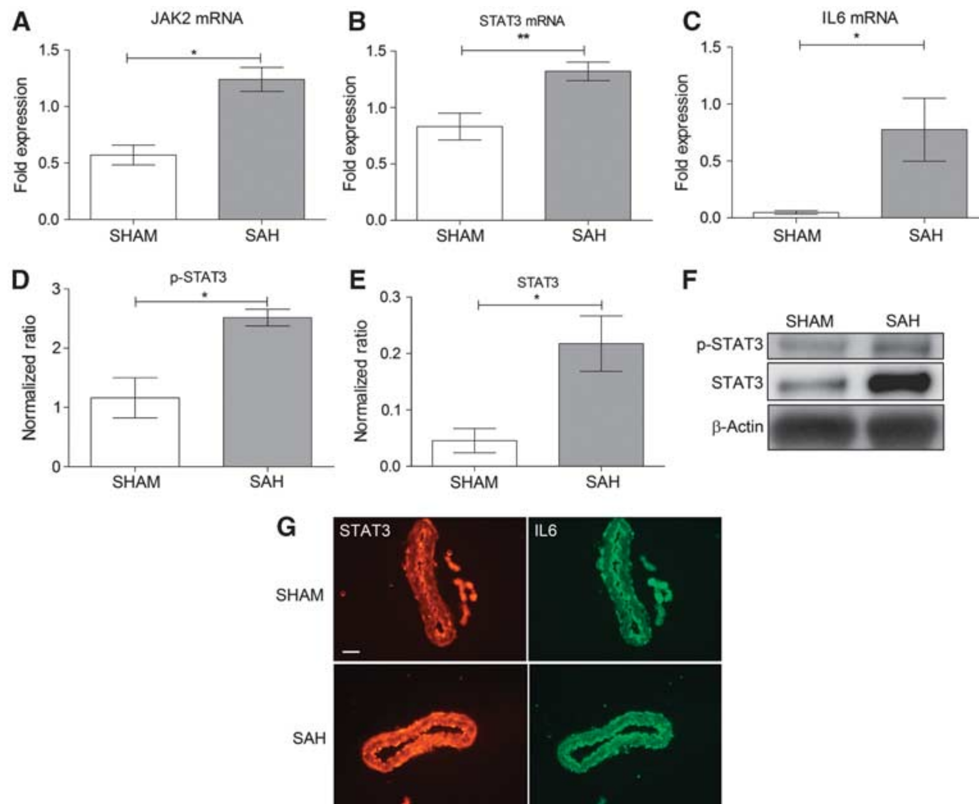

Figure 2. Change in the expression of interleukin 6 (IL-6), Janus kinase 2 (JAK2), and signal transducer and activator of transcription 3 (STAT3) after experimental subarachnoid hemorrhage (SAH). (**A–C**) IL-6, JAK2, and STAT3 mRNA expression levels at 6 hours of experimental SAH ($n = 4$). $P < 0.05$ considered as significant. (**D**, **E**) Quantification of phospho-STAT3 ($P = 0.0104$) and STAT3 ($P = 0.0338$) from western blots. Quantifications presented as means ± s.e.m. of ratio band intensity that is normalized to β-actin expression ($n = 4$). For statistical significance, *t*-test was performed between SHAM and SAH groups. $P < 0.05$ was considered as significant (*$P < 0.05$; **$P < 0.01$). (**F**) Representative immunoblots for phospho-STAT3 and STAT3. (**G**) Immunohistochemical staining showing the expression of total STAT3 and IL-6 at 6 hours of experimental SAH. Sections of middle cerebral arteries are shown as representative results of SHAM and SAH operated rats ($n = 4$, per group). The scale bar is 50 μm.

phospho-STAT3 (T705) at 6 hours of SAH. However, it diminished at later time points after SAH indicating a dispensable role in late cerebral ischemia (Supplementary Figure S1). A recent phosphoproteomic study has showed that treating SAH rats with phospho-STAT3 inhibitor had little or no effect on the neurologic outcome, which is in correlation with our observation.[17] Studies in cancer and inflammation have shown that such intracellular accumulation of STAT3 can also induce gene expression regardless of its phosphorylation status by direct DNA binding.[13,14] In agreement, we observed a significant accumulation of unphosphorylated STAT3 in the cerebral arteries of rats at 6, 24, and 48 hours after SAH (Figures 1 and 2; Supplementary Figure S2). It strongly correlates with the expression of IL-6, a primary target of STAT3. Therefore, we hypothesize STAT3 as a possible regulator of phosphorylation-independent transcription to influence expression of genes that could be responsible for sustained inflammation and late cerebral ischemia to influence the pathologic progression of SAH. Preclinical studies based on Raf/MEK/ERK1/2 inhibition by SB-386023-b or U0126 has shown reduced pro-inflammatory cytokine expression, brain infarct volume, and improved neurologic function; only when the animals were treated immediately after the insult.[5,18] No significant improvement was observed when the rats were treated at or after 6 hours post-SAH, which strongly correlates with increased accumulation of STAT3 in the cerebral arteries from the early time points after SAH. Until now, effective inhibition of STAT3-mediated transcription was achieved by inhibiting phosphorylated STAT3. Our current knowledge about small-molecule inhibitors to disrupt the unphosphorylated STAT3-mediated transcription is very limited. Therefore, a detailed study using small-molecule inhibitor directed towards unphosphorylated STAT3 is essential to establish its role in SAH pathophysiology.

It is the first study, to our knowledge, that reports increased levels of JAK-STAT signaling components (JAK2 and STAT3) in the cerebral arteries after SAH. Using system biology tools, we show STAT3 as a possible regulator of several differentially regulated genes. In the process, the study has unveiled a potential pharmacological target (STAT3) for SAH therapeutics.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support from the Lundbeck foundation for Centre of Excellence grant, Denmark, and the Swedish Heart and Lung foundation, and the Swedish Research council (grant no. 5958). We thank Dr Srinivas Veerla of SCIBLU Genomics, Lund University, Sweden, for the TFBS enrichment analysis and Dr Karin Warfvinge for the immunohistochemistry help.

REFERENCES

1 van Gijn J, Kerr RS, Rinkel GJ. Subarachnoid haemorrhage. *Lancet* 2007; **369**: 306–318.

© 2014 ISCBFM

2 Jickling GC, Xu H, Stamova B, Ander BP, Zhan X, Tian Y *et al.* Signatures of cardioembolic and large-vessel ischemic stroke. *Ann Neurol* 2010; **68**: 681–692.
3 Sriram K, Benkovic SA, Hebert MA, Miller DB, O'Callaghan JP. Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for astrogliosis *in vivo*? *J Biol Chem* 2004; **279**: 19936–19947.
4 Satriotomo I, Bowen KK, Vemuganti R. JAK2 andnd STAT3 activation contributes to neuronal damage following transient focal cerebral ischemia. *J Neurochem* 2006; **98**: 1353–1368.
5 Maddahi A, Ansar S, Chen Q, Edvinsson L. Blockade of the MEK/ERK pathway with a raf inhibitor prevents activation of pro-inflammatory mediators in cerebral arteries and reduction in cerebral blood flow after subarachnoid hemorrhage in a rat model. *J Cereb Blood Flow Metab* 2011; **31**: 144–154.
6 Veerla S, Ringner M, Hoglund M. Genome-wide transcription factor binding site/promoter databases for the analysis of gene sets and co-occurrence of transcription factor binding motifs. *BMC Genomics* 2010; **11**: 145.
7 Georgopoulos K, Winandy S, Avitahl N. The role of the Ikaros gene in lymphocyte development and homeostasis. *Annu Rev Immunol* 1997; **15**: 155–176.
8 Oyake T, Itoh K, Motohashi H, Hayashi N, Hoshino H, Nishizawa M *et al.* Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. *Mol Cell Biol* 1996; **16**: 6083–6095.
9 Hemmann U, Gerhartz C, Heesel B, Sasse J, Kurapkat G, Grotzinger J *et al.* Differential activation of acute phase response factor/Stat3 and Stat1 *via* the cytoplasmic domain of the interleukin 6 signal transducer gp130. II. Src homology SH2 domains define the specificity of stat factor activation. *J Biol Chem* 1996; **271**: 12999–13007.
10 Takizawa T, Tada T, Kitazawa K, Tanaka Y, Hongo K, Kameko M *et al.* Inflammatory cytokine cascade released by leukocytes in cerebrospinal fluid after subarachnoid hemorrhage. *Neurol Res* 2001; **23**: 724–730.
11 Lambertsen KL, Biber K, Finsen B. Inflammatory cytokines in experimental and human stroke. *J Cereb Blood Flow Metab* 2012; **32**: 1677–1698.
12 Seidel HM, Milocco LH, Lamb P, Darnell Jr. JE, Stein RB, Rosen J. Spacing of palindromic half sites as a determinant of selective STAT (signal transducers and activators of transcription) DNA binding and transcriptional activity. *Proc Natl Acad Sci USA* 1995; **92**: 3041–3045.
13 Tebbutt NC, Giraud AS, Inglese M, Jenkins B, Waring P, Clay FJ *et al.* Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. *Nat Med* 2002; **8**: 1089–1097.
14 Yang J, Liao X, Agarwal MK, Barnes L, Auron PE, Stark GR. Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NFkappaB. *Genes Dev* 2007; **21**: 1396–1408.
15 Frontera JA. Clinical trials in cardiac arrest and subarachnoid hemorrhage: lessons from the past and ideas for the future. *Stroke Res Treat* 2013; **2013**: 263974.
16 Pera J, Korostynski M, Krzyszkowski T, Czopek J, Slowik A, Dziedzic T *et al.* Gene expression profiles in human ruptured and unruptured intracranial aneurysms: what is the role of inflammation? *Stroke* 2010; **41**: 224–231.
17 Parker BL, Larsen MR, Edvinsson LI, Povlsen GK. Signal transduction in cerebral arteries after subarachnoid hemorrhage-a phosphoproteomic approach. *J Cereb Blood Flow Metab* 2013; **33**: 1259–1269.
18 Maddahi A, Edvinsson L. Enhanced expressions of microvascular smooth muscle receptors after focal cerebral ischemia occur *via* the MAPK MEK/ERK pathway. *BMC Neurosci* 2008; **9**: 85.

Supplementary Information accompanies the paper on the Journal of Cerebral Blood Flow & Metabolism website (http://www.nature.com/jcbfm)

© 2014 ISCBFM