Sphingosylphosphorylcholine is a pro-inflammatory mediator in cerebral arteries

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Running title: SPC, an inflammatory mediator in cerebral arteries

Abstract:

Inflammation plays an important role in the development of cerebral vasospasm following subarachnoid haemorrhage, however the mediators of this inflammatory response have not been clearly identified. In this study, we have investigated the potential role of two sphingolipids which occur naturally in plasma and serum, sphingosylphosphorylcholine (SPC) and sphingosine 1-phosphate (S1P), to act as pro-inflammatory mediators in cerebral artery vascular smooth muscle (VSM) cells. In rat cerebral arteries, SPC but not S1P activated p38 mitogen-activated protein kinase (MAPK). Using transcription factor arrays, two pro-inflammatory transcription factors activated by SPC in cerebral arteries were identified – nuclear factor-κB and CCAAT-enhancer binding protein. Both these transcription factors were activated by SPC in a p38MAPK-dependent manner. To determine whether this contributed to vascular inflammation, an inflammatory protein array demonstrated that SPC increased release of the chemokine, monocyte chemoattract protein-1 (MCP-1) in cultured rat VSM cells. This increase in MCP-1 expression was confirmed in cerebral arteries. S1P did not increase MCP-1 release. Taken together, our results suggest that SPC, but not S1P, can act as a pro-inflammatory mediator in cerebral arteries. This may contribute to inflammation observed following subarachnoid haemorrhage and may be part of the initiating event in vasospasm.

Key words: cerebral artery, inflammation, sphingolipid, vasospasm

Introduction:

Subarachnoid haemorrhage (SAH) occurs following the rupture of an aneurysm on the cerebral artery wall (Sehba & Bereson, 2006). This haemorrhage leads to thrombus formation on the adventitial side of the ruptured vessel and produces local ischaemia in the surrounding neural tissue (Kassel et al, 1984). In addition, in 30 - 70% of cases SAH also results in an intense and prolonged constriction of the affected cerebral vasculature (Bederson et al, 2009). This cerebral vasospasm typically occurs after a delay of 3 - 5 days post-haemorrhage reaching a maximum around 7 days. There are no efficient treatments for cerebral vasospasm and approximately 50% of SAH patients will develop a subsequent infarction (Bederson et al, 2009).

The development of cerebral vasospasm is directly linked to the presence of the subarachnoid blood clot (MacDonald & Weir, 1991). However, following blood clot formation, the processes which lead to the delayed arterial constriction are unclear (Nishizawa & Leher, 2005). Several different candidates, including oxyhaemoglobin, reactive oxygen species and the vasoconstrictor endothelin-1, have been investigated suggesting that vasospasm may be a multifactorial process (Kolias et al, 2009). Intervention studies in SAH patients who develop cerebral vasospasm have to date failed to identify a potential therapeutic target which improves patient outcome (Nishizawa & Laher, 2005; Mocco et al, 2006). Recent studies have now suggested that inflammation is an important component in the pathological development of cerebral vasospasm (Dumont et al, 2003). Analysis of cerebrospinal fluid after SAH in patients has demonstrated increases in inflammatory proteins such as interleukin (IL)-1β, IL-6 and C-reactive protein (Schoch et al, 2007; Hendryck et al, 2004). While it is expected that this inflammatory response will be at least in part produced by inflammatory cell types, gene expression studies on cerebral arteries from different animal models of SAH indicate that the vascular smooth muscle (VSM) cells also undergo an inflammatory response and likely contribute directly to inflammation during

the development of vasospasm. Elevation in the mRNA levels for IL-1β, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1 and inter-cellular adhesion molecule-1 have been observed (Onda et al, 1999; Aihara et al, 2001) in cerebral arteries following induction of SAH. Those inflammatory markers were highest at day 7 after SAH, the peak time of cerebral vasospasm. However, although inflammation of the cerebral arteries is likely to be involved, the key pro-inflammatory mediators and associated signaling mechanisms which switch on this process have yet to be identified. As vasospasm is directly related to the subarachnoid blood clot, such mediators are likely to be derived from plasma or released from blood-borne cells in serum.

Sphingolipids, produced predominantly from sphingomyelin metabolism, are now known to have important functional effects on the cardiovascular system (Alewijnse et al, 2004). Two sphingolipids, sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine (SPC), occur naturally in plasma and are elevated in serum (Yatomi, 2008; Liliom et al, 2001). The S1P concentration is approximately 300 nM in plasma and these levels appear to be regulated by erythrocytes which constitutively produce S1P (Yatomi, 2008). Less is known about the regulation of SPC (Nixon et al, 2008) although the plasma levels have been measured at 150 nM (Liliom et al, 2001). A significant proportion of plasma-derived S1P and SPC (approximately 60%) are bound to lipoproteins (Okajima, 2002). An additional source of S1P and SPC comes from activated platelets. In the case of S1P, platelets do not express S1P lyase, an enzyme responsible for the breakdown of S1P, and therefore store high concentrations of S1P (Yatomi et al, 1995). When activated, the S1P is released with other platelet-derived mediators. S1P in serum is elevated to around low μM levels. SPC is also elevated in serum (Liliom et al, 2001) but the mechanisms of this are unclear.

Both S1P and SPC engage a wide repertoire of intracellular signaling pathways in many different cell types (Sanchez & Hla, 2004; Nixon et al, 2008). It is now known that S1P-induced cellular effects occur predominantly through activation of the 7-transmembrane, G-protein-coupled receptors S1P receptors, $S1P_{1-5}$ (Sanchez & Hla, 2004). *In vitro* and S1P receptor null mice studies indicate that $S1P_1$ couples to $G_{\alpha}i$ and leads to activation of the MAPK isoform, extracellular signal-regulated protein kinase 1/2 (Sanchez & Hla, 2004). $S1P_2$ and $S1P_3$ are coupled to $G_{\alpha}q$ and $G_{\alpha}12/13$ leading predominantly to intracellular Ca^{2+} release and activation of the monomeric GTP-binding protein, RhoA (Ishii et al, 2002). The effects of $S1P_4$ and $S1P_5$ are less clear. Most cell types express at least one, and typically more than one, isoform of the S1P receptor. We have previously shown that rat cerebral artery smooth muscle cells express $S1P_{1-3}$ (Coussin et al, 2002). In addition, compared to larger blood vessels, cerebral arteries express relatively higher levels of $S1P_2$ and $S1P_3$ receptors. This greater receptor expression is probably responsible for the robust intracellular Ca^{2+} release and RhoA activation following S1P stimulation of cerebral arteries (Tosaka et al, 2001; Coussin et al 2002). Both these pathways converge on a vasoconstrictor response of the cerebral artery. Although no selective SPC receptors have been clearly identified (Nixon et al, 2008), SPC has a high structural homology with S1P and consequently may act as a lower affinity agonist at S1P receptors (Meyer zu Heringdorf et al, 2002). SPC also has a vasoconstrictor effect on cerebral arteries (Shirao et al, 2002; Mathieson & Nixon, 2006) in addition to other blood vessels such as pulmonary arteries (Thomas et al, 2005). Similar to S1P, studies have now shown that SPC has pronounced effects on cerebral vascular smooth muscle cell contractility via increases in intracellular calcium ($[Ca^{2+}]_i$) (Mathieson & Nixon, 2006) and activation of the RhoA/Rho-kinase pathway (Shirao et al, 2002). In addition, sphingolipids can activate intracellular pathways in cerebral VSM which may influence functions other than contraction. We have recently demonstrated that SPC, but not S1P, can activate p38MAPK in cerebral artery (Mathieson & Nixon, 2006). This MAPK isoform

is associated with activation of inflammatory responses (Saklatvala, 2004) and suggests that SPC may have a pro-inflammatory role in the cerebral vasculature.

Following SAH, both S1P and SPC released from platelets in the blood clot would have direct contact with cerebral VSM cells in the subarachnoid space and could potentially act as mediators of vasospasm either directly or via pro-inflammatory effects. The aim of this study was to examine the role of S1P and SPC as potential pro-inflammatory mediators in cerebral arteries. Our results demonstrate that SPC, but not S1P, can induce inflammation in cerebral artery VSM cells via activation of p38MAPK and subsequent upregulation of inflammatory transcription factors leading to release of the chemokine MCP-1. This SPC-induced process could initiate further inflammation in other cell types and, as such, may be an important component of the inflammation associated with the development of cerebral vasospasm.

Materials and Methods:

Tissue Preparation and Cell Culture

Male Sprague–Dawley rats (6 weeks old, 300–350g) were euthanised by inhalation of CO_2 followed by cervical dislocation. All procedures were in accordance with institutional guidelines. Cerebral arteries (middle and basilar) were immediately removed and placed into ice-cold physiological saline solution as previously described (Coussin et al, 2002). Dissected cerebral arteries were cleaned of connective tissue and the endothelium was removed by gentle rubbing of the arterial lumen. Cleaned arteries were pre-incubated in serum-free cell culture medium in a humidified 5% CO2 atmosphere at 37°C overnight before *ex vivo* treatment. This preincubation was necessary to ensure that the levels of MAPK phosphorylation elevated after stretch-activation of the arteries during the dissection had returned to basal levels. This was determined by preliminary experiments (data not shown). Although others have demonstrated upregulation of

receptors in rat cerebral artery smooth muscle following 24 hr organ culture (Waldsee et al, 2010), we did not observe any changes in the stimulated levels of ERK1/2 or p38MAPK. This suggests that S1P and SPC intracellular signalling were unaffected by overnight culture. The rat thoracic aorta smooth muscle cell line A7r5 (European Collection of Cell Cultures) was maintained in Dulbecco's modified eagle medium supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin. Cells were grown in a humidified 5% CO_2 atmosphere at 37°C.

Sphingolipid preparation

Lyophilised S1P and SPC (Sigma Aldrich, UK) were dissolved in methanol and stored in aliquots at -20°C. Before use methanol was evaporated and sphingolipids were dissolved in 3.6 mg/ml bovine serum albumin solution containing 10% dimethyl sulfoxide at 37°C. Concentrations of 5 μM S1P and 10 μM SPC were used throughout this study. These concentrations typically achieve maximal or close to maximal effects in concentration-effect curves as previously assessed (Coussin et al, 2002, Mathieson et al, 2006).

Transcription Factor Array

Rat cerebral arteries were incubated with 5 μM S1P or 10 μM SPC for 1 h at 37ºC. Nuclear extracts were prepared using a nuclear extraction kit (Panomics, CA, USA). Extracts were subjected to the TranSignal function-specific protein/DNA array (Panomics) according to the manufacturer's instructions to screen for changes in the binding to 20 different transcription factor binding sites (activator protein 1, CCAAT enhancer binding proteins (C/EBP), CCAAT-binding factor, cyclic AMP response element-binding protein, E4F, early growth response factors, E26 transformation-specific sequence factors, GATA-3, GATA-4, hepatocyte nuclear factor 4, heat shock factor protein, myocyte enhancer factor 2, nuclear factor of activated T-cells , YY1

transcription factor, nuclear factor-κB (NF-κB), OCT-1, peroxisome proliferator-activated receptor α, Rel, Smad binding element, Sp1). In brief, nuclear extracts were pre-incubated with biotin-labelled DNA-binding oligonucleotides to allow the formation of DNA/protein complexes. The complexes were separated from the free probes and hybridized to the TranSignal array. Signals were detected by horseradish peroxidase (HRP)-based chemiluminescence. Quantitation was performed as for immunoblots.

Immunoblotting

Rat cerebral arteries were incubated with 5 μM S1P or 10 μM SPC for the appropriate time at 37ºC. As required, arteries were additionally pre-incubated with 30 μM SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4pyridyl)1H-imidazole, a selective p38MAPK inhibitor (Calbiochem, UK) for 30 min. Treated tissue was homogenised with lysis buffer in a glass Braun homogenising vessel on ice as previously described (Coussin et al, 2002). To obtain sufficient tissue, cerebral arteries from 2-3 rats were pooled for each sample. Protein was measured using a Lowry assay (Biorad, UK) to ensure equal protein loading and membranes were stained with Ponceau Red to confirm protein loading. Protein samples were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked with 5% non-fat milk powder in tris-buffered saline, pH 7.4, containing 0.1% Tween 20. Membranes were incubated overnight at 4°C with primary antibody against I-κB (Santa Cruz Biotechnology, CA, USA), phospho-p38 (Cell Signaling, MA,USA) or GAPDH followed by HRP-conjugated secondary antibody (Sigma Aldrich). Immunoreactive bands were visualised by enhanced chemiluminescence and quantitated by densitometry as described previously (Coussin et al, 2002).

Electrophoretic mobility shift assay

Rat cerebral arteries were treated with 5 µM S1P or 10 µM SPC for 1 h at 37ºC with or without 30 min pre-incubation in 30 µM SB203580. Electrophoretic mobility shift assay (EMSA) was performed using a commercially obtained kit (Panomics) according to the manufacturer's instructions. In brief, samples were incubated with biotinylated transcription factor probe. Complexes were separated from the free probes by non-denaturing polyacrylamide gel electrophoresis. After hybridisation to Hybond-N^+ membrane, signals were detected by horseradish peroxidase (HRP)-based chemiluminescence.

Inflammatory Protein Array

A7r5 cells were treated with 5 µM S1P, 10 µM SPC or 25 µg/ml lipopolysaccharide (LPS) from Escherichia coli 0127:B8 for 24 or 48 h at 37ºC. Conditioned medium was analysed using a rat cytokine antibody array (RayBiotech, Inc., GA, USA) detecting 19 cytokines according to the manufacturer's instructions The cytokines were as follows: cytokine-induced neutrophil chemoattractant (CINC)-2, CINC-3, ciliary neurotrophic factor, fractalkine, granulocyte-macrophage-colony stimulating factor, interferon γ, IL-1α, IL-1β, IL-4, IL-6, IL-10, leukaemia inhibitory factor, leptin, MCP-1, macrophage inflammatory protein 3α, β-nerve growth factor, tissue inhibitor of metalloproteinases 1, tumor necrosis factor α, vascular endothelial growth factor. In brief, array membranes were incubated in blocking buffer for 30 min before incubation with conditioned medium for 2 h. Biotin-conjugated anti-cytokine antibodies were added for 2 h which was followed by HRP-conjugated streptavidin. After addition of detection reagent, membranes were exposed to x-ray film. Quantitation was performed using densitometry.

Enzyme-linked Immunosorbent Assay

A7r5 cells were treated with 5 µM S1P or 10 µM SPC for 24 h at 37ºC. Conditioned medium was analysed using a rat MCP-1 specific enzyme-linked immunosorbent assay (ELISA) kit

(RayBiotech, Inc). In brief, samples were added to wells coated with anti-rat MCP-1 capture antibody for 2.5 h. Biotinylated detection antibody was added for 1 h followed by HRP-conjugated streptavidin. After incubation in substrate reagent for 30 min, stop solution was added and the samples were read immediately at 450 nm using a microplate reader. For quantitation a standard curve was created using supplied recombinant rat MCP-1.

Immunofluorescence

Rat cerebral arteries were treated with either 10 µM SPC, 5 µM S1P, 1 µg/ml LPS or vehicle in the presence of 5 µg/ml brefeldin A for 48 h at 37ºC. Following stimulation rat cerebral arteries were fixed in 3% paraformaldehyde/PBS and infused with sucrose before flash-freezing as previously described (Coussin et al, 2003). Cryosectioning was performed on a Reichert Jung cryostat E / Leica CM1900 microtome at -16°C. Sections were blocked with 3% BSA/PBS before incubation with primary antibody anti-MCP-1 (Millipore, UK) overnight at 4°C. Secondary antibody FITC–anti-rabbit IgG (Jackson Immunoresearch Europe, UK) was applied for 1 h at room temperature. Subsequently nuclei were stained with BOBO-3 (Invitrogen, UK). Specificity of immunostaining was confirmed by the absence of fluorescence in arteries incubated with secondary antibody alone. Immunofluorescence was detected using a Biorad 1024 laser scanning confocal microscope (Bio-Rad) / Olympus BX50WI equipped with a krypton-argon laser and a x40 oil-immersion lens. The laser was fitted with either a blue (excitation 488 nm) or a yellow (excitation 568 nm) filter block.

Statistics

Data are expressed as mean ± S.E.M. Significance was tested by means of Student *t*-test or ANOVA where appropriate. $P<0.05$ was considered significant.

Results:

SPC but not S1P activates p38MAPK in rat cerebral arteries

We have previously demonstrated that a 15 min incubation with SPC can increase the phosphorylation of p38MAPK in denuded rat cerebral artery (Mathieson et al, 2006). This indicates an engagement of the p38MAPK signalling pathway. However, we have not previously studied the timecourse of SPC-induced activation in cerebral arteries. This could be important in relation to the sustained nature of cerebral vasospasm. Our data now show that phosphorylation of p38MAPK is activated within 5 mins and reaches a peak between 15 and 30 mins before declining towards baseline levels at 60 mins (figure 1A). S1P at 5 µM has no effect on p38MAPK phosphorylation over the timecourse of 1 hr. To ensure that the lack of effect of S1P on p38MAPK phosphorylation is due to a lower concentration (relative to SPC), we also examined the effects of 50 µM S1P. At this concentration, S1P failed to induce any increase in p38MAPK activation (data not shown).

Profile of transcription factor activity induced by SPC and S1P in rat cerebral arteries.

Due to the central role of p38MAPK in inflammatory intracellular signaling, we performed a transcription factor array to identify potential target proteins involved in sphingolipid signalling. As SPC and S1P have some divergence of intracellular signaling, we were particularly interested in transcription factor activity where S1P and SPC had different effects. While this preliminary screen was limited to 20 transcription factor binding sequences, it provided information regarding the potential downstream effects of SPC and S1P. Rat cerebral arteries were treated *ex vivo* with either 5 µM S1P or 10 µM SPC for 1 h at 37ºC. Nuclear extracts were then subjected to the assay procedure. Of the 20 consensus sequences contained in the array, 5 revealed a change in DNA binding which was considered to be potentially significant (using an arbitrary cut-off of an

approximately 2-fold increase or decrease). Only 2 consensus sequences demonstrated a difference in DNA binding for S1P compared to SPC. These oligonucleotide sequences demonstrated the binding of NF-κB and C/EBP. Binding to both these oligonucleotides was increased by incubation with SPC but unchanged by incubation with S1P (figure 1B). These transcription factors are closely associated with inflammation and were investigated further.

Activation of the NF-κB signalling pathways by SPC but not S1P

To confirm preliminary findings of the transcription factor arrays, we conducted further experiments on the activation of NF-κB. The activity of the transcription factor NF-κB is regulated in several ways (Hayden et al, 2006). The best established model proposes that the inhibitor of NF-κB, IκB, sequesters NF-κB in the cytoplasm of unstimulated cells and thus maintains the transcription factor in an inactive state. Upon phosphorylation by IκB kinase, the phosphorylated IκB becomes ubiquitinated and subsequently degraded. This effectively releases NF-κB which subsequently translocates to the nucleus and initiates expression of target genes. We initially examined the activation of this signaling complex by determining the degradation of IκBα, an indirect indicator for activation of the NF-κB signalling pathway. Rat cerebral arteries were incubated *ex vivo* with either 5 μM S1P or 10 μM SPC for the appropriate time at 37ºC. Tissue protein extracts were then subjected to immunoblot analysis with antibodies detecting the α-subunit of the IκB complex. In a timecourse of SPC incubation, IκBα expression was unchanged at 5 min but decreased maximally between 15 and 30 min and returned towards baseline at 60 min (figure 2A). S1P had no effect on IκBα expression (figure 2B). To determine the role of p38MAPK in the SPC-induced degradation of IκBα, cerebral arteries were pre-incubated with SB203580 for 30 mins prior to incubation with SPC for 15 mins. SPC-induced degradation of IκBα was significantly inhibited by SB203580. As p38MAPK may have an important role in SPC-induced NF-κB activation, we examined the binding of activated NF-κB to

consensus sequences using EMSA. Rat cerebral arteries were treated ex vivo with S1P or SPC for 1 h at 37ºC with or without pre-treatment with 30 μM SB203580 as above. Subsequently nuclear extracts were subjected to EMSA. SPC, but not S1P, incubation increased NF-κB binding to NF-κB consensus sequences (figure 2C). This effect of SPC was prevented by pre-incubation with the p38MAPK inhibitor.

Activation of the C/EBP pathway by SPC but not S1P

The transcription factor array indicated that SPC could activate the pro-inflammatory transcription factor C/EBP in rat cerebral arteries. The potential binding of C/EBP to consensus oligonucleotide sequence was examined by EMSA as above. Rat cerebral arteries were treated with S1P or SPC for 1 h at 37ºC with or without pre-treatment with SB203580 and nuclear extracts prepared. SPC, but not S1P, increased C/EBP binding to DNA (figure 3). This effect of SPC was diminished by p38MAPK inhibition.

SPC-induced inflammatory protein production in VSM cells

As pro-inflammatory signalling pathways were activated in cerebral artery following SPC incubation, we investigated the downstream consequences. Cytokine protein arrays were used to identify release of candidate inflammatory proteins. Due to the technical limitations of rat cerebral artery (tissue yields are insufficient, even after substantial pooling from multiple animals to measure release of inflammatory proteins) the rat A7r5 thoracic aorta smooth muscle cell line was used as an *in vitro* model. To confirm that this was an appropriate model for rat cerebral artery, A7r5 cells were initially examined to determine if SPC could induce NF-κB and C/EBP activation. EMSA was performed as above for rat cerebral artery. Following 1 hr incubation with 10 μM SPC, an increase in DNA binding to oligonucleotides encoding consensus sequences for NF-κB and C/EBP was observed (figure 4A). This was blocked by pre-incubation with 30 μM

SB203580 for 30 mins. S1P did not increase DNA binding (data not shown). These results with A7r5 cells are similar to those obtained with rat cerebral artery.

To determine cytokine release A7r5 cells were firstly treated with 10 μM SPC or 5 μM S1P for 24 h or 48 h at 37ºC. Conditioned medium was analysed using a cytokine protein array according to the manufacturer's instructions. Lipopolysaccharide (LPS, 1 μg/ml) was used as a positive control. In LPS-treated A7r5 cells the release of most of the cytokines detected by the array was increased as expected. The most pronounced increases were observed for interleukin-6, CINC-3, fractalkine, granulocyte-macrophage-colony stimulating factor, MCP-1 and macrophage inflammatory protein 3α. However, incubation with SPC for either 24 or 48 hours increased the release of only one inflammatory protein, the chemokine monocyte chemoattract-1 (MCP-1) (figure 4B). S1P did not produce an increased release in any inflammatory proteins detected by the array.

To confirm results with the rat cytokine array, ELISA was performed using A7r5 cells. Cells incubated with 10 μM SPC for 24 hours had a significant increased release of MCP-1 (approximately 3 fold compared to control) (figure 4C). LPS increased MCP-1 release by approximately 15 fold.

Once MCP-1 release in A7r5 cells was established, we tested whether *ex vivo* treatment with SPC elicited MCP-1 production in rat cerebral artery. Arteries were treated with either 10 μM SPC or 1 μg/ml LPS in the presence of 5 μg/ml brefeldin A for 48 h at 37ºC. Brefeldin A blocks secretory pathways of the Golgi system and was used to accumulate MCP-1 inside VSM cells. This allowed detection of MCP-1 production in small tissue samples. MCP-1 production was assessed by immunofluorescence. LPS, which served as positive control, induced MCP-1 production inside

cerebral artery VSM cells (figure 5). This staining pattern was limited to the cytoplasm and was generally a patchy distribution. This probably reflected the distribution in organelles, presumably the golgi system. SPC incubation also induced an increase in MCP-1 staining of a similar pattern to LPS.

Discussion:

A potential role for sphingolipids in the development of vasospasm has previously been suggested via contractile effects on cerebral arteries. We (Coussin et al, 2002; Mathieson et al, 2006), and others (Shirao et al, 2002), have demonstrated that S1P and SPC can induce a constriction of cerebral arteries *in vitro* by directly activating intracellular contractile processes. This has been further demonstrated by *in vivo* administration of S1P or SPC directly to the cisterna magna in animal models (Tosaka et al, 2001; Kurokawa et al, 2009). The timecourse of vasospasm in these *in vivo* studies is maximal approximately 2 hours after application and decreases thereafter. As this timecourse differs from the clinical profile of vasospasm, a maintained constriction would probably also require other mechanisms to be activated. Several studies now show that inflammation could contribute to the maintained vasoconstriction of the cerebral arteries (Dumont et al, 2003). Our previous study has demonstrated that, in addition to the contractile effect, SPC can also activate the MAPK family members, p38MAPK (Mathieson et al, 2006). This kinase is closely associated with pro-inflammatory intracellular pathways (Saklatvala, 2004). We now reveal in the current study that SPC, but not S1P, can activate the pro-inflammatory transcription factors NF κB and C/EBP in cerebral artery VSM cells. This occurs via SPC-induced activation of p38MAPK. Activation of these transcription factors leads to upregulation of the chemokine MCP-1.

The findings presented in the current study are only of potential relevance to vasospasm if SPC levels are increased during SAH. A recent clinical study has demonstrated that in patients who have developed SAH the concentration of SPC in the cerebral spinal fluid (CSF) is significantly increased approximately 17 fold after 7 days (Kurokawa et al, 2009). This provides direct evidence that SPC is elevated in SAH and could therefore contribute to vasospasm. The mechanisms of this increase are not known. It is likely that increased levels of SPC are due to release from activated platelets (Liliom et al, 2001). However, in SAH it has also been shown that erythrocytes are an important component in the development of vasospasm (Macdonald et al, 1991). While it has been reported that S1P accumulates in erthyrocytes (Yatomi, 2008), SPC metabolism in erthrocytes has not been assessed. As both S1P and SPC are increased in serum, it remains a possibility. Sphingolipids are therefore probably increased in the subarachnoid space by several different pathways within the area of a cerebral aneurysm. In the current study we have used maximal, or just below maximal, concentrations of S1P and SPC (as assessed by concentration-effect curves previously in rat cerebral arteries)(Coussin et al, 2002; Mathieson et al, 2006). These are in the range typically used by many other studies in different cell types. Although the concentrations of SPC measured from CSF *in vivo* are significantly lower (in the order of 30 nM, Kurokawa et al, 2009) than that used in our study, it is notable that SPC levels decrease rapidly in CSF from animal models (Kurokawa et al, 2009). The relevant concentrations *in vivo* in this case are not clear, and local concentrations are likely to be much higher than current estimates in serum. Certainly, low μM levels could be possible in SAH.

Although inflammation may have a primary role in cerebral vasospasm, the pro-inflammatory mediators have not yet been clearly identified. A recent study has demonstrated that cerebral ischaemia itself can contribute to a localized inflammation (Maddahi & Edvinsson, 2010) which may play a role in SAH pathology. In the current study, the ability of SPC to upregulate MCP-1

in a reasonably selective manner (at least in the rat A7r5 VSM cell line) suggests a potentially specific role for this sphingolipid in the inflammation associated with vasospasm following blood clot formation. Although we cannot state that this selectivity occurs in the rat cerebral artery, microarray experiments similar to that performed by Vikman et al (2007) to determine inflammatory gene expression could provide further evidence of this. Interestingly, MCP-1 is upregulated in the CSF of patients with SAH (Gaetani et al, 1998) and may be of particular relevance to clinical vasospasm. A recent study has suggested that MCP-1 levels are directly correlated with an outcome of angiographically demonstrated vasospasm and could therefore be a biomarker for predicting onset (Kim et al, 2008). In an experimental model of SAH, MCP-1 expression was increased in cerebral artery VSM cells (Lu et al, 2009) demonstrating that the VSM cells could directly contribute to the observed increase in MCP-1 levels. Our data now provide a potential novel mediator, naturally occurring in plasma and serum, which can increase MCP-1 expression in rat cerebral arteries. Our results also delineate an intracellular mechanism for the SPC-induced increase in MCP-1 expression. We now demonstrate that in cerebral arteries, SPC, via activation of p38MAPK, can increase the activity of two pro-inflammatory transcription factors, NF-κB and C/EBP. This leads to enhanced specific DNA binding. Both these transcription factors are closely associated with an increase in inflammatory cytokines and chemokines in VSM cells (Sekine et al, 2002; Dwarakanath et al, 2004). Although NF-κB and C/EBP belong to families of transcription factors, identifying the specific isoforms of each transcription factor involved in SPC-mediated MCP-1 expression is beyond the scope of this study. The MCP-1 gene promoter region contains promoter sites for both C/EBP and NF-κB (Sekine et al, 2002; Ueda et al, 1997).

Other inflammatory proteins, in addition to MCP-1, are upregulated during SAH and likely also contribute to the inflammation associated with vasospasm. The SPC-induced effects are therefore

likely to be part of a larger inflammatory response involving multiple cell types and intracellular pathways. With regard to VSM cells, it is also possible that S1P (which did not lead to increased expression of any inflammatory proteins in this study) may have other actions which have not yet been characterized. It is also acknowledged that the main inflammatory cells in SAH are likely to be leukocytes which will drive the increase in inflammatory mediators. However, SPC could have an important role in initiating this response by stimulating the infiltration of monocytes into the vessels wall via expression of MCP-1. Therefore, although the SPC-induced MCP-1 expression is unlikely to contribute to the majority of inflammation, it may be sufficient to play an important and primary role shortly following SAH. Until the site of action of SPC in cells is uncovered and SPC pharmacology further developed, it will be difficult to confirm the relative importance of this sphingolipid. Although SPC can act as a low affinity agonist at S1P receptors, the divergence of SPC and S1P signaling observed in our studies (current study and Mathieson et al, 2006) indicate that this is not the case in cerebral arteries. Preliminary studies with the limited S1P receptor antagonists available had no effect on SPC-induced MAPK activation (data not shown). A direct intracellular action of SPC also cannot be ruled out.

In conclusion, our study has demonstrated that SPC can act as a pro-inflammatory mediator in cerebral artery VSM cells by upregulating expression of the chemokine, MCP-1. The mechanism of this upregulation occurs through activation of pro-inflammatory transcription factors. This provides evidence that SPC is a novel link between blood clot formation and the initiation of inflammation. Further *in vivo* studies are now required to demonstrate the pathological role of SPC as a pro-inflammatory mediator following SAH. This could prove to be a useful therapeutic target to combat cerebral vasospasm.

Disclosure/conflict of interest:

None of the authors have any disclosure or conflict of interest.

Supplementary information is available at the *Journal of Cerebral Blood Flow and Metabolism* website.

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Titles and legends to figures:

Figure 1

SPC activates p38MAPK and the transcription factors NF-κB and C/EBP.

(**A**) Rat cerebral arteries were treated *ex vivo* with 5 µM S1P or 10 µM SPC over a time course of 60 min. A representative immunoblot of phospho-p38MAPK shows time-dependent SPC-induced increase in phosphorylation with a maximum between 15 and 30 min (n=3). S1P did not change phospho-p38MAPK levels. GAPDH served as protein loading control. (**B**) Rat cerebral arteries were treated as above for 1 h. Nuclear extracts were screened for transcription factor activity using a transcription factor array. SPC increased DNA binding to NF-κB and C/EBP consensus sequences (n=2). S1P had no effect on DNA binding to the same sequences.

Figure 2

Activation of the NF-κB signalling pathway by SPC is dependent on p38MAPK.

(**A**) Rat cerebral arteries were treated *ex vivo* with 10 µM SPC over a time course of 1 h with or without pre-incubation in 30 µM SB203580 (a p38MAPK inhibitor) for 30 min. A representative immunoblot shows a time-dependent decrease of IκBα levels with a minimum between 15 and 30 min. GAPDH served as protein loading control (n=6). (**B**) Rat cerebral arteries were incubated as above for 15 min following pre-incubation with 30 µM SB203580 for 30 min. A representative immunoblot and mean data demonstrate that the SPC-induced reduction of IκBα expression was reversed by pre-incubation with the p38MAPK inhibitor (* $P<0.05$, n=4). S1P had no effect on IκBα expression. (**C**) Nuclear fractions of cerebral arteries incubated in SPC or S1P as above for 1 h following pre-incubation with 30 µM SB203580 were subjected to EMSA. A representative EMSA shows that SPC but not S1P treatment increased binding to oligonucleotides specific for NF-κB consensus sequence (n=3). This was diminished by p38 inhibition. Specific binding

induced by SPC was decreased to control levels following inclusion of cold probe. SB203580 alone had no effect on DNA binding.

Figure 3

Activation of C/EBP by SPC is dependent on p38MAPK.

Nuclear fractions of cerebral arteries incubated in 10 µM SPC or 5 µM S1P as above for 1 h following pre-incubation with 30 µM SB203580 were subjected to EMSA and the binding of C/EBP to DNA consensus sequences was determined. Incubation with SPC increased C/EBP binding to specific oligonucleotides. This effect of SPC was decreased by p38 inhibition. S1P did not increase C/EBP activity. SB203580 alone also had no effect on DNA binding to this oligonucleotide. A representative EMSA is shown (n=3).

Figure 4

SPC induces MCP-1 release in A7r5 cells.

(**A**) A7r5 cells derived from neonatal rat aorta were incubated in 10 µM SPC for 1 h following pre-incubation with 30 µM SB203580 for 30 min. Representative EMSA of nuclear extracts shows increased NF-κB and C/EBP binding to respective consensus sequence oligonucleotides. Increased DNA binding of both transcription factors by SPC was inhibited by pre-incubation of the p38MAPK inhibitor. (**B**) A7r5 cells were treated with 5 µM S1P, 10 µM SPC or 25 µg/ml LPS for 24 or 48 h at 37ºC. Conditioned medium was analysed using a cytokine protein array (n=3, representative blots shown at 48 hr timepoint of specific cytokines detected). LPS increased expression of most of the cytokines detected by the array (data not shown except example of IL-6 release). SPC increased the release of only MCP-1 but no other cytokines. No cytokine release was observed with S1P. There was no difference between 24 or 48 h timepoints (**C**) To confirm array results, A7r5 cells were treated with 10 µM SPC for 24 h at 37ºC and conditioned medium

was analysed using a MCP-1-specific ELISA. SPC incubation significantly increased MCP-1 release from A7r5 cells compared to S1P (* $P<0.05$, n=4–12). The positive control, LPS, also increased MCP-1 release as expected (n = 2).

Figure 5

SPC induces MCP-1 production in rat cerebral artery VSM cells.

Rat cerebral arteries were treated *ex vivo* with 10 µM SPC or 1 µg/ml LPS in the presence of 5 µg/ml brefeldin A for 24 h or 48 h. Arterial sections were co-stained with anti-MCP-1 antibody (green) and nuclei stained postfix with BOBO-3 (red). Immunofluorescent images demonstrate that SPC induced MCP-1 production in cerebral artery VSM cells and this was localized to the cytoplasm. LPS also increased MCP-1 in a similar localization pattern. There was no difference between 24 and 48 h sections. Typical immunostained sections shown from at least four different cerebral arteries. Scale denotes 5 µm.

Figure 1

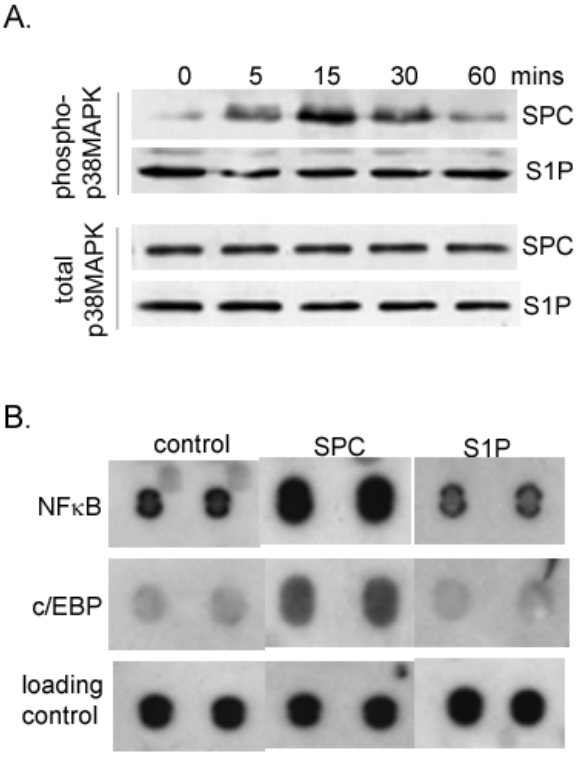

A.
0
5
15
30
60 mins
phospho-p38MAPK
SPC
S1P
total p38MAPK
SPC
S1P
B.
control
SPC
S1P
NFκB
c/EBP
loading control

Figure 2

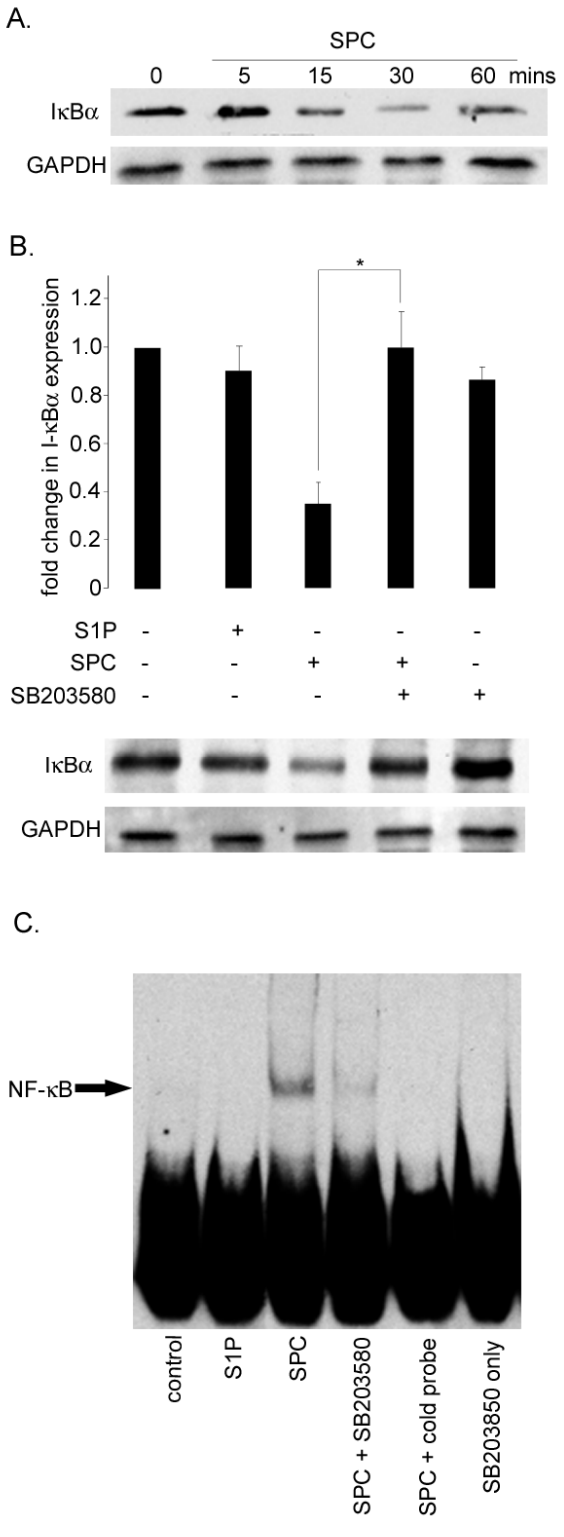

A.
SPC
0
5
15
30
60 mins
IκBα
GAPDH
B.
*
fold change in I-κBα expression
1.2
1.0
0.8
0.6
0.4
0.2
0
S1P - + - - -
SPC - - + + -
SB203580 - - - + +
IκBα
GAPDH
C.
NF-κB
control
S1P
SPC
SPC + SB203580
SPC + cold probe
SB203850 only

Figure 3

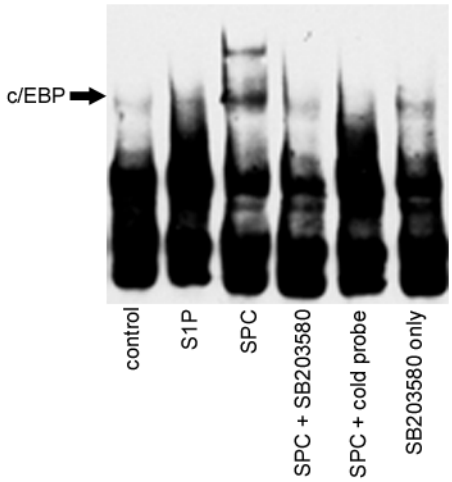
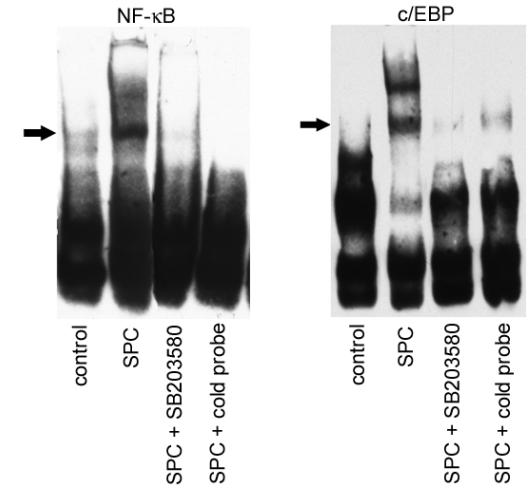
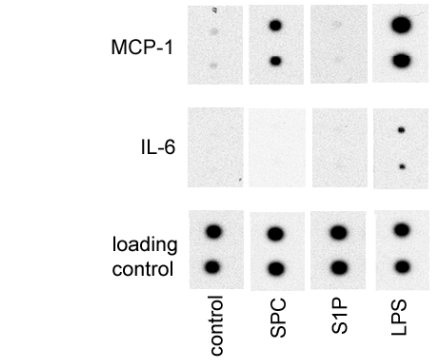

c/EBP
control
S1P
SPC
SPC + SB203580
SPC + cold probe
SB203580 only

Figure 4


A.
NF-κB
c/EBP
control
SPC
SPC + SB203580
SPC + cold probe
control
SPC
SPC + SB203580
SPC + cold probe


B.
MCP-1
IL-6
loading control
control
SPC
S1P
LPS

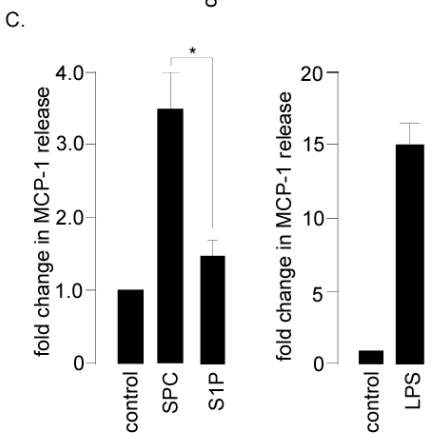

C.
fold change in MCP-1 release
4.0
3.0
2.0
1.0
0
*
control
SPC
S1P
fold change in MCP-1 release
20
15
10
5
0
control
LPS

Figure 5