Cerebral ischemia induces transcription of inflammatory and extracellular matrix related genes in rat cerebral arteries

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Running title: MAPK and inflammation following cerebral ischemia

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Abstract

Background and purpose – Cerebral ischemia results in a local inflammatory response that contributes to the size of the lesion, however, the involvement of the cerebral vasculature in unknown. We hypothesise that the expression of inflammatory genes (Il6, iNOS, cxcl2, TNF-α and Il-1β) and extracellular-matrix-related genes (MMP9, MMP13) is induced in cerebral arteries following cerebral ischemia via activation of mitogen activated kinases (MAPKs).

Methods – This hypothesis was tested in vivo by experimental subarachnoid haemorrhage (SAH) and temporal middle cerebral artery occlusion (MCAO), and by organ culture of isolated cerebral arteries with quantitative real time PCR (mRNA expression) and immunohistochemistry (localization of protein expression). The gene promotors were investigated in silica with computer analysis.

Results – The mRNA analysis revealed that the ischemic models, SAH and MCAO, as well as organ culture of isolated cerebral arteries resulted in transcriptional upregulation of the abovementioned genes. The protein expression involved phosphorylation of three different MAPKs signalling pathways (p38, ERK 1/2 and SAPK/JNK) and the downstream transcription factors (ATF-2, Elk-1, c-Jun) shown by immunohistochemistry and quantified by image analysis. All three models revealed the same pattern of activation in the cerebrovascular smooth muscle cells. The in silica analysis demonstrated binding sites for said transcription factors.

Conclusions - The results suggest that cerebral ischemia and organ culture induce activation of p38, ERK 1/2 and SAPK/JNK in cerebral arteries which in turn activate the transcription factors ATF-2, Elk-1 and c-Jun and the expression of inflammatory and extracellular-matrix-related genes in the wall of cerebral arteries.

Introduction

Despite considerable advances in the knowledge of molecular mechanisms involved in cerebral ischemia the morbidity and mortality of cerebral ischemia remain high. Stroke, either thromboembolic or hemorrhagic, is associated with reduction in cerebral blood flow and cerebral metabolism, resulting in cerebral ischemia, apoptosis and ultimately ending in cell death.[1] Much research on mechanisms involved in cerebral ischemia has been focused on cytotoxic and apoptotic events that occur in the ischemic core and in the penumbral region; however, little attention has been directed towards the cerebral vessel walls. Since it is well known that cytokines and growth factors contribute to the cellular damage seen following a stroke we have addressed the question if there is an associated local inflammatory response in the cerebral artery walls that might be part of the remodelling seen in the ischemic artery following a stroke. This view is based on two previous microarray studies on rat and man providing support for this suggestion.[2, 3]

We hypothesise that cerebral ischemia induces expression of genes related to inflammation (cytokines, chemokines and adhesion molecules) and to the extracellular matrix in cerebral arteries. To test this suggestion we used two in vivo models (experimental subarachnoid haemorrhage, SAH, and temporal middle cerebral artery occlusion, MCAO), and in vitro by organ culture of isolated cerebral artery segments. The activation of inflammatory/extracellular matrix genes were quantified by real-time PCR and the protein expression of signal transduction pathways examined with immunocytochemistry and image analysis. In-depth analysis of the molecular mechanisms responsible for arterial gene regulation was tested using a

mitogen activated protein kinase (MAPK) inhibitor in the organ culture model. We observed regulation of metalloproteinase (MMP) 9 and 13, inducible NO synthase (iNOS), interleukin (Il) 6 and the chemokine cxcl2, genes previously seen implied in the arterial response.[2, 3] This was associated with activation of the MAPK signalling pathways, p38, ERK 1/2 and SAPK/JNK and their downstream transcription factors ATF-2, Elk-1 and c-Jun in the cerebral arteries. To verify that the induction of pro-inflammatory genes are not only correlative to the activation of MAPK signalling a specific ERK1/2 inhibitor was used and found to attenuate the transcriptional increase.

Materials and Methods

Vessel preparation for organ culture

Male Sprague Dawley rats (n=48) weighing 250-350g were euthanized by CO_2 and decapitated. The middle cerebral (MCA) and basilar (BA) arteries were carefully dissected out with intact endothelium. These were used for incubation and placed in Dulbecco´s Modified Eagle Medium (DMEM) and incubated at 37ºC with 4% CO_2 for either 0, 6 or 24 hours.[4, 5] In a subset of experiments the MAPK ERK1/2 inhibitor U0126 (10μM) was included in the incubation medium (see Henriksson et al 2004).[6] In organ culture experiments controls were fresh vessel segments; in conjunction with the ERK1/2 experiments where vessels incubated with vehicle (DMSO) for the same time was used as control. Following this the arteries were instantaneously frozen and stored at -80ºC prior to being studied with molecular techniques. All the experiments were approved by the Ethics committee of the University (case number M137-05).

Rat subarachnoid haemorrhage model

Male Sprague-Dawley rats (n=24) weighing 350-400 g were anesthetized using 5% halothane (Halocarbon Laboratories, River Edge, New Jersey) in N_2O/O_2 (70:30). SAH was induced by injection of 250 µl blood, removed from the tail artery, intracranially at a pressure equal to the mean arterial blood pressure (80-100 mmHg). Sham rats received identical treatment but injection of blood was omitted since preliminary studies[7] have shown that both the elevation of intracranial pressure and the blood *per se* contribute to about an equal extent to the ischemia that occurs after SAH. For a detailed description see Beg et al[8]. During the period of observation, the rat was monitored regularly and if it showed severe distress was killed prematurely (3 % mortality). Following the procedure described the MCA and the basilar arteries were removed after 24 hours and snap frozen in liquid nitrogen.

Middle cerebral artery occlusion

Male Wistar Hannover rats weighing 350-400 g were obtained from Harlan, Horst, Netherlands (n=24) and used because this rat type has a suitable anatomy[9]. The animals were housed under controlled temperature and humidity conditions with free access to water and food. The experimental procedures were approved by the Animal Ethics Committee of Lund University (M131-03). A middle cerebral artery occlusion (MCAO) was induced by an intraluminal filament technique, previously described by Memezawa et al[9]. Anaesthesia was induced using 4.5% halothane in N_2O:O_2 (70%:30%) and kept anesthetized by inhalation of 1.5% halothane through a mask. For a detailed description see Stenman et al[10]. The MCA was removed bilaterally, the ipsilateral as involving cerebral ischemia and the contralateral as a control[4, 5].

Previous studies have shown that fresh removed arteries had the same expression as the contralateral artery. The arteries were then treated as below.

RNA isolation

Following the manufacturer's protocol, total RNA preparations were obtained using the Trizol RNA isolation kit (Invitrogen, Sweden). For more details see Vikman et al [11]. The RNA was then resuspended in 10 μl of nuclease free water and the 260/280 values were measured by means of a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden). All RNA was tested for genomic DNA contamination with the real-time PCR primers.

Real-Time PCR investigation

The cDNA for the real-time PCR reactions were created in 40 μl reactions using superscript III in accordance with the manufacturer's instructions (Perkin-Elmer, Applied Biosystems). One μg of RNA was used in each reaction. The subsequent real-time PCR was performed in a GeneAmp 5700 Sequence Detection system (Perkin-Elmer, Applied Biosystems) using the GeneAmp SYBR® Green kit (Perkin-Elmer, Applied Biosystems) with a 25 μl reaction volume and one μl of the abovementioned cDNA serving as a template. Each reaction was performed in duplicate and was run for 40 cycles. β-actin, elongation factor 1 (EF-1) and GAPDH were used as housekeeping genes. The gene expressions were normalised versus the housekeeping genes to account for differences in the starting material and in the cDNA reaction efficiency; for a more detailed account of the procedure see [12]. Each primer pair was specific for the genes of interest (Table 1) and the primers were, when possible, designed to span an exon-exon boundary.

Immunohistochemistry

The rat MCA was dissected out, treated as described above and placed in Tissue TEK (Gibco), frozen and subsequently sectioned into 10 μm thick slices in a calibrated Microm HM500M cryostat (Microm, Germany). The primary antibodies used were rabbit antiphospho p38 (Cellsignalling #4631), rabbit antiphospho ERK 1/2 MAPK (Cellsignalling, #4376), rabbit antiphospho SAPK/JNK (Cellsignalling, #9251) rabbit antiphospho ATF-2 (Cellsignalling #9221), rabbit antiphospho Elk-1 (Cellsignalling, #9181) and rabbit antiphospho c-Jun (Cellsignalling, #9261) each diluted 1:50. The secondary antibodies used were biotin-conjugated goat anti-rabbit antibodies (JackssonImmuno, 111-065-003) diluted 1:100. All dilutions were with PBS containing 0.3% Triton-X100 and 5% fetal calf serum. The Vectastain ABC kits (Vector Laboratories) together with DAB substrate (Rockland) were used for detection in accordance with the manufacturers' instructions. Pictures were taken at 40X magnification. Secondary antibodies alone served as a control.

Picture analysis

We investigated the fluorescence or DAB intensity using ImageJ (http://rsb.info.nih.gov/ij/). Each sample was investigated in four preset regions blindly to the treatment and the sample intensity defined as the mean of these four regions. Each measurement was done in equal sized areas and the sample mean values were used in calculating the mean at each point in time (n=4 animals in each group).

Promotor analysis

To find the putative promoters, we used the computer program BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), a program that compares selected sequences and looks for homologies. The mRNA sequences for the above mentioned genes were compared with the entire rat genome. The promoter was defined then as being 1500 base pairs upstream from the start codon, AUG. In the case of AUG being placed in the second exon, it was defined instead as being 1500 base pairs upstream from the first exon. The promotors obtained were subsequently investigated with the program MATCH (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi), that looks for known transcription factor binding sites in selected sequences. This program compares any given sequence with a matrix that contains sequences selected for known transcription factors. We used a pre-made matrix containing binding sites for vertebrate transcription factors alone for finding the binding sites in our putative promotors. Only high quality matrices were employed. The cut-off was set to minimize the error rate for both false positives and false negatives.

Statistics

For the organ culture experiments differences were investigated using the Kruskal-Wallis test with Dunn´s test which allows for comparisons between more than two groups. The Mann-Whitney test was used for the SAH and MCAO real-time PCR comparisons which allows for the straight comparison between two groups ($p<0.05$ being regarded as significant expression).

Results

mRNA expression in cerebral arteries

A. Organ culture

Based on a previous microarray study of gene expression following SAH[11] we chose to investigate the expression of the positively regulated genes following organ culture (0, 6 and 24 hours). Gene expression was normalised to the expression of three different housekeeping genes: β-actin, EF-1 and GAPDH. All the housekeeping genes gave the same result after normalisation in MCA (Figure 1) and basilar arteries (data not shown). Results are presented as mean regulation ± S.E.M. in comparison with 0 hours, which was set as the baseline. This was done for all the genes except for Il6 which did not show any expression at 0 hours; accordingly, we used the results at 6 hours for statistical comparison for this gene. In the MCA the inflammatory genes Il6, iNOS, cxcl2, TNF-α and Il-1β were significantly upregulated following organ culture. The levels at 0 hours were very low for all the five inflammatory genes with a peak at 6 hours and a lower but still sustained expression at 24 hours; Il-1β showed a continuing increase during the 24 hours period (Figure 1). The extracellular matrix regulating genes MMP9 and MMP13 showed increased expression following organ culture. For MMP9 the expression at 6 hours largely remained for up to 24 hours whereas for MMP13 it continued to rise at 24 hours (Figure 1d-e). When investigating the gene expression in the basilar artery we observed the same pattern of regulation as in the MCA (results not shown).

B. Subarachnoid haemorrhage

The expression of the inflammatory genes (Il6, iNOS, cxcl2, TNF-α and Il-1β) and the extracellular-matrix-related genes (MMP9 and MMP13) in the cerebral vessels were studied at 24 hours following SAH. Expression of the inflammatory and extracellular-matrix-related genes increased in comparison to 0 hours (Figure 2). In the SAH studies EF-1 and β-actin were used for normalisation in this study since GAPDH expression has been shown to be unstable following SAH[13]. The level of expression at 24 hours is compared with that of 0 hours (Figure 2). Since there was no expression of Il6 prior to SAH, the data presented in the figure shows the expression of this gene as the mean ± S.E.M. without normalisation. There was significant upregulation of all genes examined; TNF-α, Il-1β, Il6, iNOS, cxc12, MMP9 and MMP13 (Figure 2).

C. Middle cerebral artery occlusion (MCAO)

The expression of four of the inflammatory genes that were investigated (Il6, cxcl2, TNF-α and Il-1β) showed upregulation in the MCA investigated following MCAO. The expression of iNOS did not change following focal ischemia. The extracellular-matrix-related genes that were investigated, MMP9 and MMP13, showed increased levels of expression as compared with the fresh arteries (Figure 3) and with the contralateral non-ischemic side (data not shown).

In comparing the data from the present investigation we observed that all of the genes investigated, with the exception of iNOS, were regulated in similar fashion following organ culture and cerebral ischemia.

Activated signalling pathways

A. Organ culture

The activated, phosphorylated forms of the MAPK signal transducers (p38, ERK 1/2, SAPK/JNK) and their downstream transcription factors (ATF-2, Elk-1, c-Jun) were investigated using immunohistochemistry with antibodies specific for their phosphorylated forms. There was no activation of p38 in fresh vessels but there was activation at 6 hours and this further increased to 24 hours of organ culture (Figure 4 a-c). ERK 1/2 shared this pattern with no activation at 0 hours with a marked increase in activation at 6 and 24 hours (Figure 4 d-f). The pattern of activation for SAPK/JNK was similar showing a continued increase at 6 and 24 hours (Figure 4 g-i). Three transcription factors were chosen for investigation based on promoter analysis (see below). There was no activation of ATF-2 in fresh vessel segments whereas there was some phosphorylated ATF-2 at 6 hours and strong activation at 24 hours (Figure 5 a-c). Phosphorylated Elk-1 appeared already in fresh vessels, with increased activation at 6 and 24 hours (Figure 5 d-f). The c-Jun phosphorylation was strongest at 6 hours with levels basically remaining high at 24 hours. This clearly shows the presence of activated transcription factors in the nucleus of the smooth muscle cells indicating an active role in the transcriptional activation (Table 2).

B. Middle cerebral artery occlusion

There was activation of the p38, ERK1/2 and SAPK/JNK signalling pathways in cerebral arteries leading to the ischemic region after MCAO. It was observed that, following MCAO p38 was activated in the ipsilateral MCA of the rats following MCAO whereas there was no activation in the fresh vessels (Figure 6 a,d) and only a weak activation in the contralateral arteries (data not shown). This was also true of

ERK 1/2 (Figure 6 b, e) and SAPK/JNK (Figure 6 c, f), which showed an increase in phosphorylation following MCAO but no activation in fresh arteries (Table 2). p38, ERK 1/2 and SAPK/JNK showed weak activation in arteries in the contralateral non-ischemic side (results not shown). Since this activation was weak as compared with the ipsilateral side we used fresh arteries to illustrate the difference (Figure 6). We also observed strong significant phosphorylation of the downstream transcription factors ATF-2 (Figure 6 g, j) and c-Jun (Figure 6 i, l) with no activation in the fresh arteries. There was a weak baseline activation of Elk-1 (Figure 6 hours, k) on the ipsilateral side (Table 2). ATF-2, Elk-1 and c-Jun showed weak activation on the contralateral side (results not shown).

C. Subarachnoid haemorrhage

The data regarding the MAPKs and the transcription-factors were highly similar with regard to the regulation seen in after MCAO (Table 2). Comparison of the data regarding signal transduction activation in the cerebral arteries following SAH and focal ischemia with organ culture of isolated cerebral artery segments also showed strong similarity. This implies that the ischemic models and the organ culture activate the MAPKs studied and their downstream transcription factors in a similar fashion (Table 2).

ERK1/2 inhibition

Co-incubation during organ culture of isolated cerebral artery segments with U0126 (10 μM) or vehicle revealed that U0126 but not vehicle caused a disappearance of ERK1/2 activity and of the transcription factor Elk-1. (Figure 6). In addition, the previously noted increase in expression of inflammatory and extracellular matrix

related genes (see above) was attenuated (data not shown). This implies that the Elk-1 activation is primarily through ERK1/2 signalling rather then p38 and that said activation in part is responsible for the increase in transcription.

Promotor analysis

The putative promoter regions of the inflammatory and extracellular-matrix-related genes were investigated for the presence of ATF-2, Elk-1 and c-Jun binding sites (Table 3). MMP13, cxcl2 and TNF-α all contained Elk-1 sites, iNOS and Il6 contained both Elk-1 and ATF-2 sites whereas MMP9 and Il-1β contained neither Elk-1 nor ATF-2 sites (Table 3). However, all the genes that were studied contained binding sites for c-Jun. It was concluded that the activated transcription factors investigated can bind and activate transcription in the inflammatory and extracellular-matrix-related genes, although the binding sites involved can differ.

Discussion

This is the first study to demonstrate that in two ischemic models in vivo and during organ culture in vitro there are similarities in the regulation of inflammatory and extracellular-matrix-related genes in the cerebrovascular smooth muscle. We have in addition demonstrated the pattern of activation of the MAPKs p38, ERK1/2 and SAPK/JNK together with their downstream transcription factors ATF-2, Elk-1 and c-Jun are activated in a similar manner after SAH, MCAO and organ culture. Treatment with an Erk1/2 inhibitor in the organ culture model resulted in the absence of activation of all the above signals. In addition, the promoter analysis revealed that the genes investigated have binding sites for said transcription factors, demonstrating that the activation can affect the transcription of these genes. We therefore

hypothesise that changes that occur in conjunction with cerebral ischemia activates the MAPK signalling pathways thereby resulting in increased transcription of the inflammatory and extracellular-matrix-related genes in the cerebral artery wall.

There is a plethora of studies describing the involvement of inflammation, apoptosis and cell death after cerebral ischemia[14-17]. There are, however, to the best of our knowledge no previous reports regarding increased expression of inflammatory genes (Il6, cxcl2 and iNOS) or extracellular-matrix-related genes (MMP9 and MMP13) in cerebral arteries following cerebral ischemia. The genes presently chosen for studies were based on an initial observation by microarray that revealed upregulation in cerebral arteries following SAH [2, 3, 5, 11] while TNF-α and Il-1β were selected due to recent reports of their importance following cerebral ischemia[14-17]. It was observed that in all three models there were highly similar expression profiles for the genes examined. The extracellular events that triggered activation differed, however. We hypothesise that one major causative factor is the change in shear stress which is caused by (i) the rise in intracranial pressure and the subsequent reduction in cerebral blood flow and wall tension in SAH, (ii) the temporary blockage of perfusion in the MCAO, and (iii) the removal of the intralumnial pressure during the organ culture procedure. Since mechanical stress has been reported to activate MAPKs[18], these events could result in the activation of upstream signal transducers (p38, ERK 1/2 and SAPK/JNK) and of the transcription factors (ATF-2, Elk-1 and c-Jun). To verify this activation, we examined the activation/phosphorylation of three major MAPKs; p38, ERK 1/2 and SAPK/JNK and their downstream transcription factors. The pathways chosen were based on previously reported signs of inflammation in stroke [19-21].. In the promoter analysis the genes were found to have binding sites for some or all of the

transcription factors studied, making them putative targets, further emphasising the importance of pinpointing the signalling pathways (Table 3). This *in silica* analysis of the promotors was limited to the transcription factors investigated using immunohistochemistry. We performed this investigation since there are no precise data regarding the promotors of said genes even though there are reports supporting our data; e.g. Moon *et al* showed that AP-1 sites increase the transcription of MMP9 [22] while Selvamurugan *et al* and Mangshol *et al* observed the same for MMP13.[23-25] It is worth mentioning that such an activation is never straight forward; there is always the potential for synergistic effects between the different pathways. The activation of a single transcription factor might yield no effect unless it is supported by other activated transcription factors and these signaling pathways yield different results in different cell types.[18]

The activation of MAPKs is known to increase the expression of inflammatory and extracellular-matrix-related genes in brain tissue and participate in inflammatory processes[20, 21]. The novelty of our findings is that the MAPKs were found to be activated in smooth muscle cells of the arteries after cerebral ischemia. We have previously shown that ERK 1/2 is activated after organ culture.[6] In the present study we demonstrate that ERK 1/2 is activated at 24 hours after SAH or MCAO in cerebral arteries. In the present study we have revealed that not only is the ERK 1/2 inhibited by U0126 but so is the subsequent phosphorylation of Elk-1. Furthermore, the mRNA expressions of the inflammatory and extracellular matrix genes are blunted. The importance of this pathway has been illustrated by use of ERK1/2 inhibitors which attenuate cerebral blood flow reduction following SAH and brain tissue damage

following MCAO.[8, 26]. In addition there is phosphorylation of p38 and SAPK/JNK after cerebral ischemia and organ culture, however this occur at a later stage and is not particularly strong. This demonstrates again the consistency of MAPK activation in cerebral arteries in our model of cerebral ischemia and organ culture. Investigating the activation of the downstream transcription factors (ATF-2, Elk-1 and c-Jun) revealed phosphorylation of these transcription factors as well, revealing phosphorylation of targets downstream of the MAPKs investigated. Thus, the results support the hypothesis of differential activation of p38, ERK 1/2 and SAPK/JNK pathways in cerebral arteries after cerebral ischemia and increased transcription of the investigated inflammatory and extracellular-matrix-related genes.

Previous studies have revealed that cerebral ischemia (SAH and MCAO) and organ culture result in enhanced expression of endothelin, angiotensin and serotonin receptors in the arterial wall at both a molecular and a functional levels.[4, 10, 12, 27-29] The receptor upregulation and the enhanced expression of inflammatory and extracellular-matrix-related genes in cerebral ischemia and in organ culture are supported both by the present results of signal transduction and gene regulation and by results from studies of upregulation of G-protein coupled receptors in rat[10, 12, 27, 28] and in man [5, 30]. We therefore suggest (i) that organ culture can be utilised as a way of studying molecular events in cerebral arteries that may occur following cerebral ischemia, and that (ii) there is similar mechanisms activated during ischemia and organ culture of cerebral arteries.

The investigation of cerebral arteries revealed that there is activation of the signal transducers p38, ERK 1/2, SAPK/JNK and their downstream transcription factors ATF-2, Elk-1 and c-Jun. Molecular studies have demonstrated a number of other

transcription factors that are activated by these MAPKs, however, we had no technical possibility to study all these. The promoters of the inflammatory and extracellular-matrix-related genes contain binding sites for the transcription factors investigated. Interestingly the same is true of the promotors used by the G-protein coupled receptors found previously to be upregulated after organ culture (ET_A, ET_B, AT_1, AT_2, 5-$HT_{2A/1B/1D}$). This study show that the activation of MAPKs are indeed important for the increase in transcription seen for these inflammatory and extra-cellular-matrix related genes. Further studies are needed thought to fully clarify the importance and cross talk of said signalling pathways in the transcriptional regulation.

Conclusion

This is the first study to conclusively demonstrate that organ culture of rat cerebral arteries results in molecular events that resemble those that occur after cerebral ischemia in brain arteries in vivo. The increase in transcription of inflammatory and extracellular-matrix-related genes in the cerebrovascular smooth muscle cells appearently use the same pathways. Thus, the arterial response observed following cerebral ischemia and that found in cerebral arteries after organ culture have a high degree of similarity, and the organ culture approach may be used to investigate molecular mechanisms shared in cerebral ischemia.

Figure 1. Gene regulation following organ culture in MCA. All samples are normalised versus 0 hours. The values are expressed as mean ± S.E.M, n=6 in each group, *=p<0.05 as compared with 0 hours.

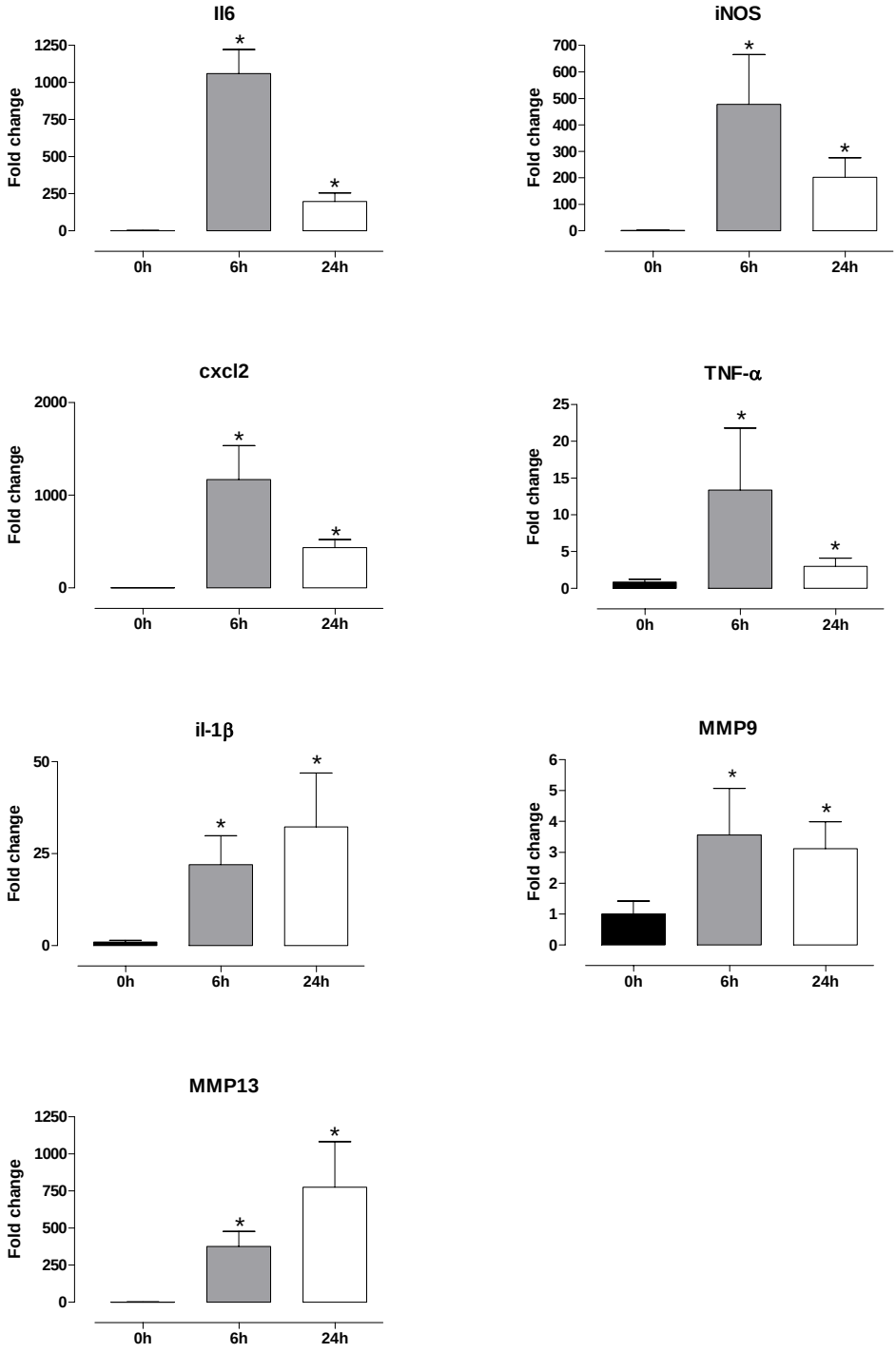

Figure 2. Gene regulation following SAH in cerebral arteries. All samples are normalised versus 0 hours except for Il6, which was not normalised. Values are expressed as mean ± S.E.M, n=6 in both groups, *=p<0.05 as compared with 0 hours.

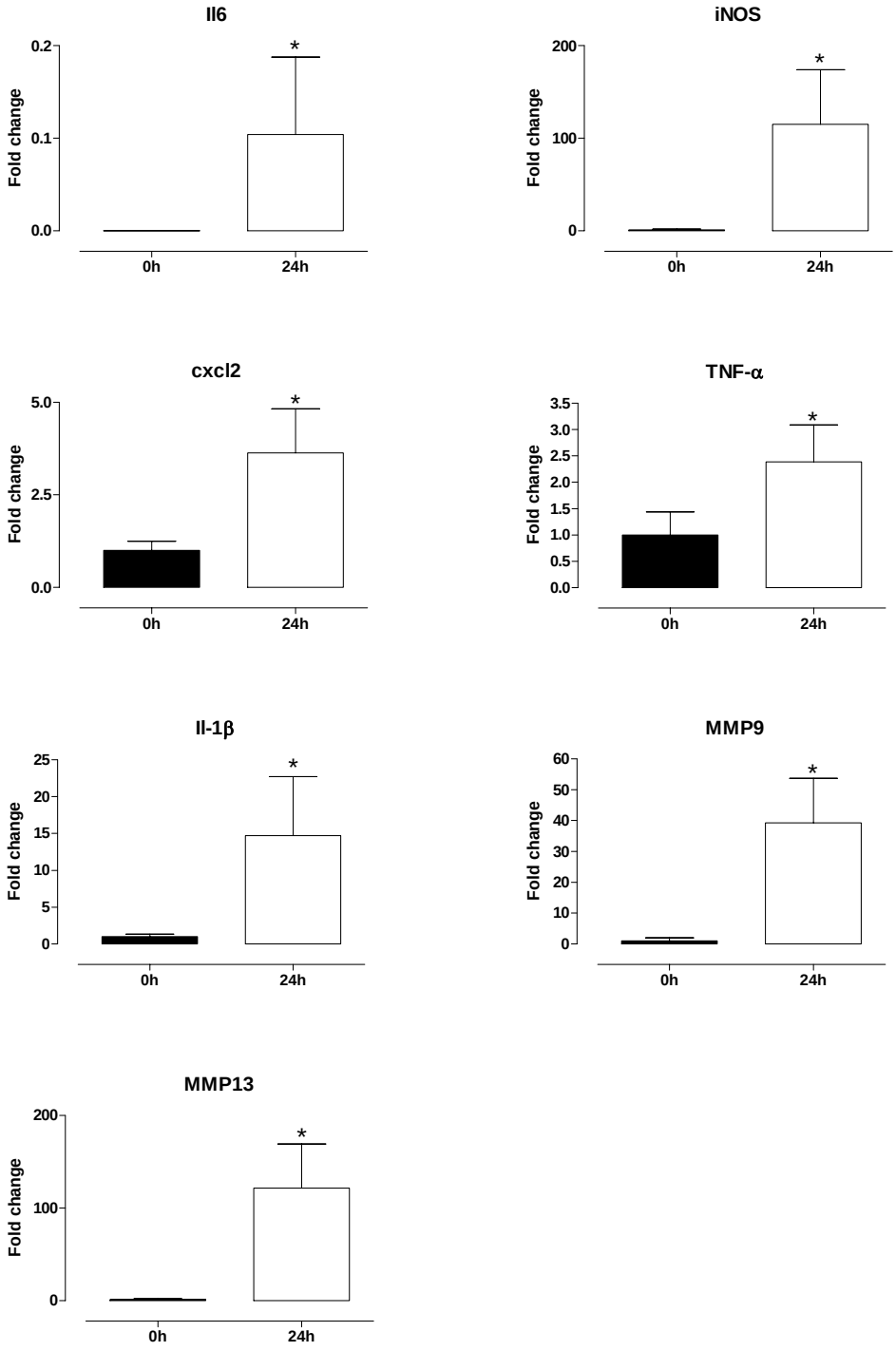

Figure 3. Investigation of inflammatory and extracellular matrix related genes in focal ischemia revealed there to be upregulation of all the genes except for iNOS, for which no change in its expression was found. The values are expressed as mean ± S.E.M, n=6 in each group, *=p<0.05 as compared with 0 hours.

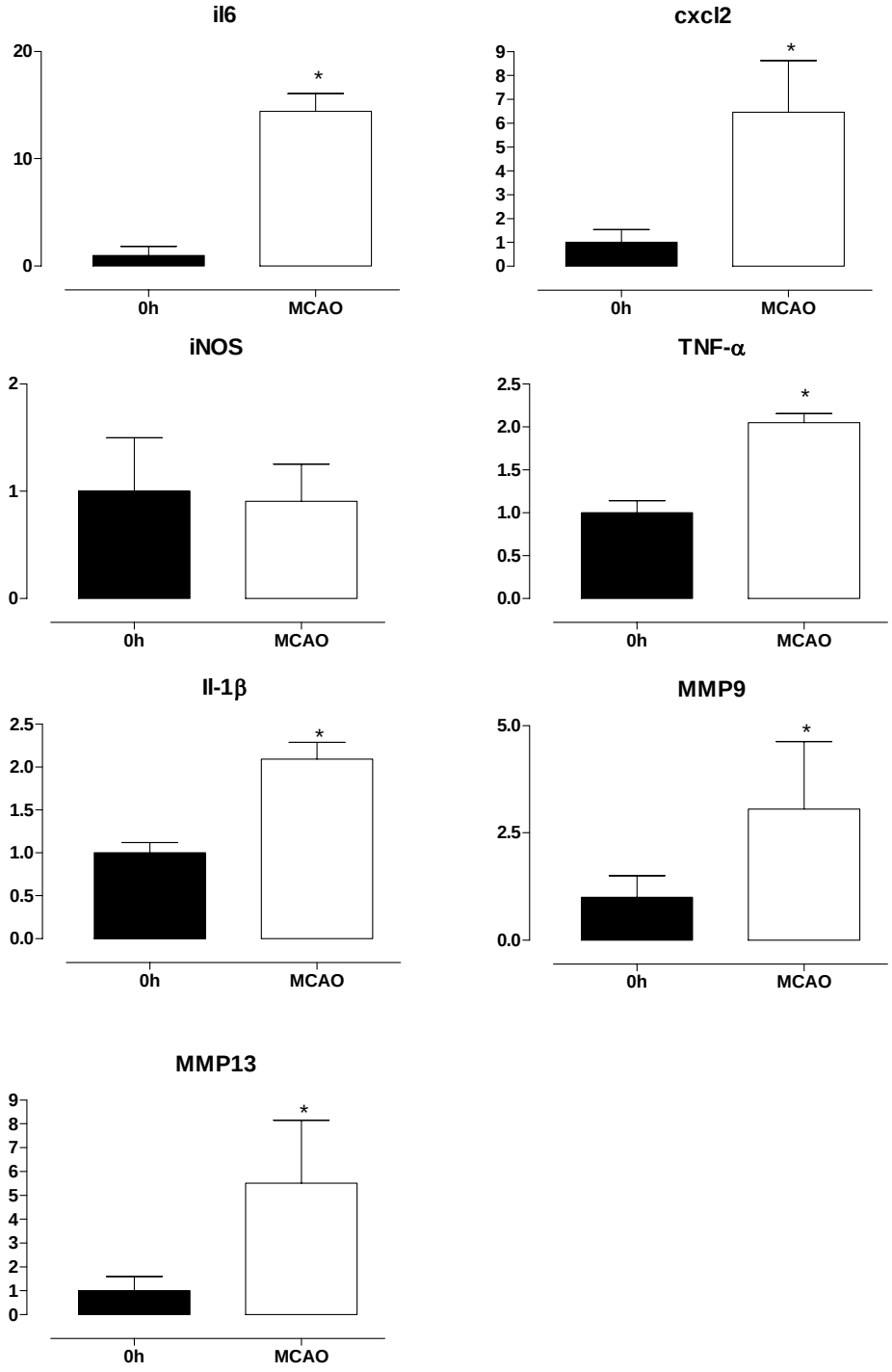

Figure 4. There was no activation of p38 in fresh vessels (0 hours) whereas there was a light activation of it at 6 hours, the activation increasing up to 24 hours during organ culture (a-c). For ERK1/2 there was no phosphorylation at 0 hours but there was a strong activation of it at 6 hours and 24 hours (d-f). For SAPK/JNK there was a gradual increase from 6 to 24 hours (g-i).

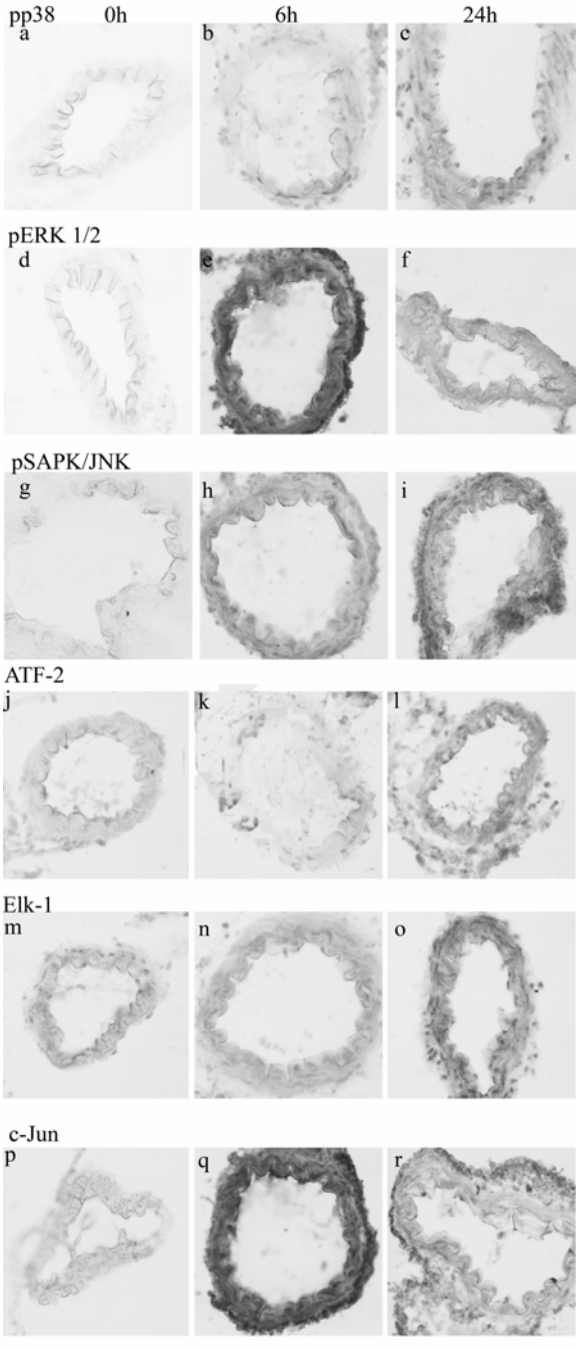

Figure 5. There was no phosphorylation of ATF-2 or c-Jun in fresh vessels (0 hours) whereas there were already some phosphorylated Elk-1 present in the fresh vessels. Weak ATF-2 phosphorylation was observed at 6 hours and a strong activation at 24 hours of organ culture (a-c). There was a slight increase in Elk-1 phosphorylation at 6 hours which increased to 24 hours. Phosphorylation of c-Jun was strongest at 6 hours and decreased slightly at 24 hours.

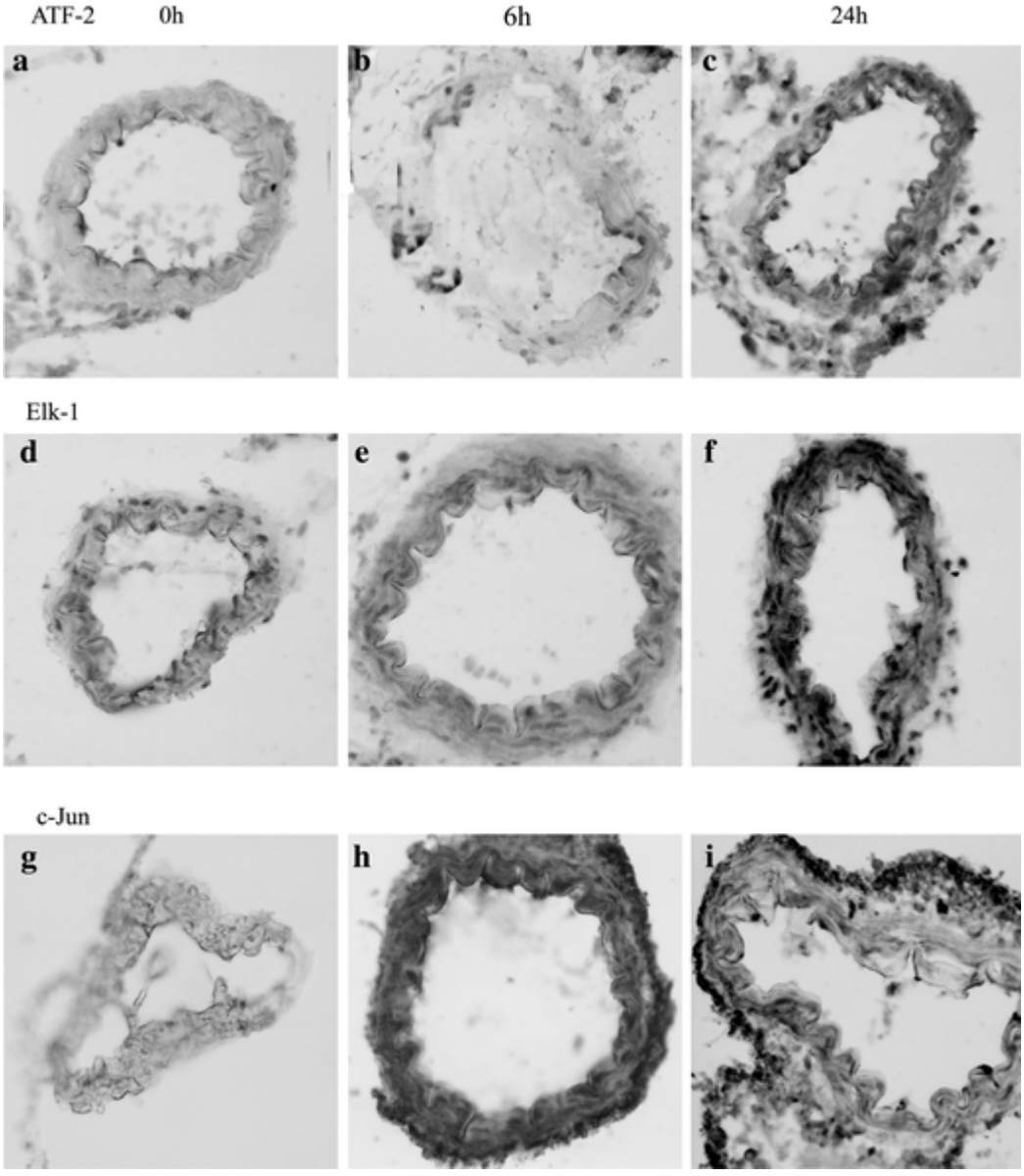

Figure 6. Immunohistochemical examination of MCA segments from the ischemic side of MCAO after 24 hours compared with controls. A strong p38 activation in the smooth muscle cells of MCA of rats was observed following MCAO whereas there was no activation in fresh vessels (a,d). The same pattern was observed for ERK1/2 (Figure b, e) and SAPK/JNK (c, f) which showed an increase in phosphorylation following MCAO and an absence of activation in the fresh arteries. Analysis of the downstream transcription factors revealed an increase in the phosphorylation of ATF-2, Elk-1 and c-Jun. ATF-2 (g, j) and c-Jun (,i, l) showed in the fresh arteries a lack of activation, whereas a was weak endogenous activation of Elk-1 was noted (h, k).

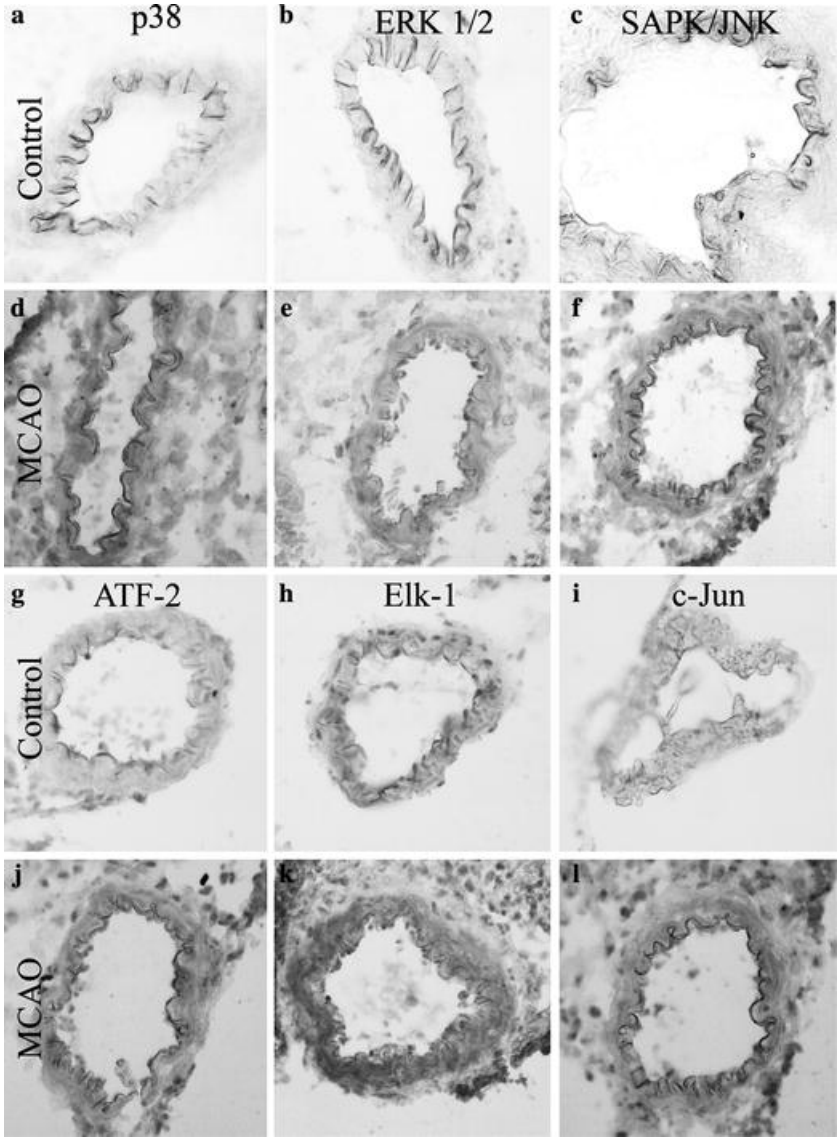

Figure 7. The treatment of arteries with U0126 removed the activation of ERK 1/2 and Elk-1 while the treatment with vehicle (DMSO) alone did not affect said activation. This indicates that ERK 1/2 activation is the main factor for Elk-1 activation rather then p38.

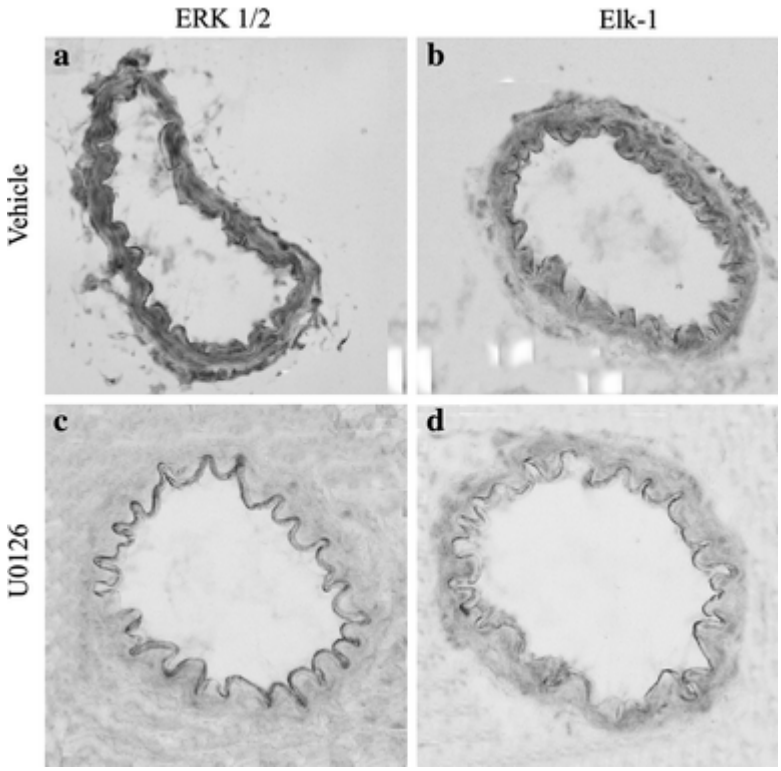

Table 1. Accession numbers and primer sequence for the genes that were investigated.

Gene name	Abbreviation	Accession number	Primer sequence
beta-actin	ACTB	NM_031144	5' GTAGCCATCCAGGCTGTGTTG 3'
			5' TGCCAGTGGTACGACCAGAG 3'
Elongatin factor 1	EF-1	NM_175838	5' GCAAGCCCATGTGTGTTGAA 3'
			5' TGATGACACCCACAGCAACTG 3'
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	AB017801	5' GGCCTTCCGTGTTCCTACC 3'
			5' CGGCATGTCAGATCCACAAC 3'
Interleukin 6	Il6	NM_012589	5' AAGAGACTTCCAGCCAGTTGCC 3'
			5' ACTGGTCTGTTGTGGGTGGTATC 3'
Induible NO synthase	iNOS	NM_012611	5' CAATGGCTTGAGGCAGAAGC 3'
			5' GCCACCTCGGATATCTCTTGC 3'
Chemokine (C-X-C motif) ligand 2	cxcl2	NM_053647	5' TTGTCTCAACCCTGAAGCCC 3'
			5' TGCCCGTTGAGGTACAGGAG 3'
Matrix metalloproteinase 9	MMP9	NM_031055	5' AAGCCTTGGTGTGGCACGAC 3'
			5' TGGAAATACGCAGGGTTTGC 3'
Matrix metalloproteinase 13	MMP13	M60616	5' TCTGCACCCTCAGCAGGTTG 3'
			5' CATGAGGTCTCGGGATGGATG 3'
tumor necrosis factor alpha	TNF-α	NM_012675	5' AAAGCATGATCCGAGATGTG 3'
			5' AGCAGGAATGAGAAGAGGCT 3'
interleukin 1 beta	Il-1β	NM_031512	5' TTGTGCAAGTGTCTGAAGCA 3'
			5' TGTCAGCCTCAAAGAACAGG 3'

Table 2. The signalling pathways found to be active (phosphorylated) after SAH, organ culture and MCAO at 24 hours. Note that p38, ERK 1/2 and SAPK/JNK were all active just as their downstream transcription factors were as well. Values are given as mean ± S.E.M, n=6, *=p<0.05

Signalling Transducer	Organ culture	SAH	MCAO
pp38	3.3±0.55	2.4±0.26	6.0±0.14
pERK 1/2	4.4±0.93	2.4±0.37	3.5±0.26
pSAPK/JNK	3.1±0.35	2.9±0.26	4.7±0.14
pATF-2	2.0±0.35	2.1±0.32	1.8±0.06
pElk-1	1.7±0.1	2.4±0.33	1.6±0.24
pc-Jun	3.0±0.27	2.1±0.39	1.8±0.06

Table 3. Promotor analysis revealing there to be binding sites for ATF-2, Elk-1 and c-Jun in the promoters that were analysed. Activation of the transcription factors and the subsequent increase in transcription indicate these pathways to be active and increasing the transcription of the genes in question.

	Number of binding sites		
Activated by	ERK 1/2 or p38	p38 or SAPK/JNK	SAPK/JNK
Transcription factor binding site	Elk-1	ATF-2	c-Jun
Il6	1	7	7
iNOS	2	6	10
cxcl2	3	0	3
MMP9	0	0	11
MMP13	2	0	2
TNF-α	1	0	4
Il-1β	0	0	3

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