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Sexual Dimorphism in Gene Expression after Aneurysmal Subarachnoid Hemorrhage

Victor Friedrich, PhD[2], **Weina Bi, MD**[1], and **Fatima A. Sehba, PhD**[1,2,*]

[1]Department of Neurosurgery, Icahn School of Medicine at Mount Sinai, New York, New York

[2]Department of Neuroscience, Icahn School of Medicine at Mount Sinai, New York, New York

Abstract

Background and Purpose—Inflammation and compromise in structure and function of cerebral parenchymal microvasculature begins early after subarachnoid hemorrhage (SAH). We recently found greater inflammation and greater vascular compromise in male than in female rats following SAH. In this study we investigated whether this cross-sexual difference in pathology is reflected in expression levels of genes related to vascular inflammation and structural compromise.

Method—Age-matched male and female rats underwent sham surgery or SAH by endovascular perforation. Early physiology (intracranial pressure (ICP), blood pressure (BP), heart rate (HR) and cerebral blood flow (CBF)) was monitored. Cerebral RNA was extracted at sacrifice 3 hours after surgery and assayed for expression of thrombomodulin (Thbd), endothelial nitric oxide synthase (eNos;Nos3), intracellular adhesion molecule-1 (Icam1), vascular endothelial growth factor (Vegf), interleukin-1beta (Il1β) tumor necrosis factor-alpha (Tnf-α) and arginine vasopressin (Avp).

Results—Increases in ICP and BP at SAH appeared slightly greater in males but the difference did not reach statistical difference, indicating that SAH intensity did not differ significantly between the sexes. Of the seven genes studied two; Tnf-α and Vegf, did not change after injury while the remainder showed significant responses to SAH. Response of Nos3 and Thbd was markedly different between the sexes, with expression greater in males.

Conclusion—This study finds that sexual dimorphism is present in the response of some but not all genes to SAH. Since products of genes exhibiting sexual dimorphism have anti-inflammatory activities, our results indicate that previously found sex-based differences in vascular pathology are paralleled by sexually dimorphic changes in gene expression following SAH.

Keywords

Gender; early brain injury; vascular pathology; inflammation; stroke

[*]*Corresponding Author*: Fatima. A. Sehba, Department of Neurosurgery, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1136, New York, NY 10029-6574. fatima.sehba@mssm.edu Phone: 212 241 6504, Fax: 212 241 0697.

Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is *sexually dimorphic* in prevalence and age of attack but not in outcome. Women harbor more cerebral aneurysms and have a greater incidence of SAH. Remarkably, although the average age of female victims of SAH is greater than that of male victims the two groups experience similar outcomes.[1, 2] We previously hypothesized that this similarity in outcomes reflects sex-based differences in pathophysiology associated with SAH, and examined the influence of sex on acute physiology and early brain injury in an experimental model. We found that the rise in intracranial pressure and peripheral blood pressure at SAH was greater in males than in same-aged females, and that microvessel pathology and neuronal apoptosis were greater in the males than in the females.[3] The present study extends these observations by examining sexual dimorphism in gene expression after SAH. The study includes five genes known previously to increase early after SAH, endothelial nitric oxide synthase (Nos3), intracellular adhesion molecule-1 (Icam1), interleukin-1beta (Il1β), tumor necrosis factor-alpha (Tnf-α), and vascular endothelial growth factor (Vegf), and in addition two genes known to increase early after ischemic stroke, thrombomodulin (Thbd), and vasopressin (Avp).[4–7]

We found that five of those genes respond to SAH, and the response of two was sexually dimorphic. Interestingly, those genes that showed sexually dimorphic expression, with expression greater in males, encode products that oppose vascular inflammation. Taken together our data indicate that previously found sex-based differences in vascular pathology are paralleled by sex-related differences in gene expression following SAH.

Methods

All experimental procedures and protocols used in this study were reviewed and approved by the Animal Care Committee of the Icahn School of Medicine at Mount Sinai.

Surgical preparation, physiological monitoring and SAH production

SAH was induced in three-month-old male (408.6 ± 5.5 g) and female (299 ± 5.0 g) Sprague Dawley rats (N=9 per sex) using the endovascular suture model.[8] Briefly, rats were anesthetized with ketamine-xylazine (50mg/Kg+5mg/Kg IP), transorally intubated, and positioned in a stereotactic frame. Thereafter, ventilation and anesthesia were maintained by inspired isoflurane (1-2% in 21% oxygen-supplemented room air) and body temperature was maintained at 37°C by a homeothermic blanket (Harvard Apparatus) and a rectal temperature probe. The right femoral artery was exposed and cannulated for *blood gas and blood pressure monitoring (BP).* The atlanto-occipital membrane was exposed and cannulated for *measurement of intracranial pressure (ICP).* A Laser Doppler Flowmetry (LDF) probe (0.8mm diameter, model P-433, Vasamedics Inc.) was placed adjacent to the coronal suture and 5 mm lateral to the right of midline, over the territory of the middle cerebral artery and away from large meningeal vessels for *cerebral blood flow (CBF) measurement.*

SAH was induced by advancing a suture retrogradely through the ligated right external carotid artery (ECA), and then distally through the internal carotid artery (ICA), until the

suture perforated the intracranial bifurcation of the ICA. This event was confirmed by a rapid rise in ICP and a decrease in CBF.[9, 10] Following surgery, each animal was returned to its cage as it regained consciousness and was able to breathe spontaneously. Sex- and time-matched sham operated animals (N=9 per sex) were used as controls in this study. Animals were sacrificed 3 hours after hemorrhage or sham surgery. As described previously, sham surgery included all steps carried for SAH induction, except that perforation of the internal carotid artery was not effected [11]. Animals were randomized for gender and type of surgery (SAH or sham).

Data Acquisition and Statistical Analysis

CBF, ICP, and BP data were recorded continuously from 20 minutes before to 60 minutes after SAH using PolyView software (Grass Instruments; MS, USA).[8] Heart rate (HR) was computed from the recorded BP data.[12] BP, CBF and HR data were normalized to baseline values defined as average values over the 20 minutes prior to SAH, and responses to surgery were analyzed as percentage of baseline. Statistical analysis was performed using two-way ANOVA followed by multiple Fisher's PLSD *post-hoc* t-tests setting experimental significance at $p<0.05$.

Reverse transcription polymerase chain reaction (rtPCR)

RNA extraction and rtPCR was performed in the Quantitative PCR (qPCR) CORE at Icahn School of Medicine at Mount Sinai by staff members who were blind to the identity of specimens.

Primers—Primers (Table 1) were designed using the BLAST program and were purchased from Eurofins MWG Operon (Alabama, USA).

RNA extraction—Rats were transcardially perfused with chilled saline and brains were rapidly removed and frozen (2 methylbutane on dry ice). Coronal brain slices spanning bregma −8.0 to +1.2 [13] were cut and immediately placed in RNeasy (Qiagen). RNA was isolated from brain tissue using RNeasy Lipid mini kit following the manufacturer's protocol (QIAGEN) and its quantity, quality and purity but not integrity were assessed (optical density (OD) 260, and OD ratios 260/280 and 260/230, NanoDrop).

rtPCR—cDNA was synthesized from total RNA with AffinityScript™ Multi-Temp RT (Agilent Technologies) with oligo dT as primer. rtPCR was performed using PlatinumTaq DNA polymerase (Invitrogen) and SYBR® green (Molecular Probes) with an Applied Biosystems thermocycler (ABI7900HT). The PCR conditions used were: 95° for 2 min, 40 cycles of 95° for 15s, 55° for 15s, 72° for 30s. RNA levels for the housekeeping gene tubulin was also assayed in all samples and was used as internal control. Relative fold mRNA levels were determined by the $2^{-\Delta\Delta CT}$ (cycle threshold) method (ABI) and the results (“copy number”) are the ratios of test gene to housekeeping gene mRNA.

Statistical analysis was performed by multivariate (StatView v. 5.0.1, SAS Institute Inc. USA) using a design crossed in sex and treatment (sham, SAH) with interaction and nested in animal. Results showed significant effects in hemorrhage ($F(1,16)= 64.1$, $p= 0.0001$) and

interaction terms (Sex*Hemorrhage: F(1,16)=6.34, p=0.022). Consequently, univariate ANOVAs were performed on individual genes, with pairwise comparisons performed by Fisher's PLSD post-hoc t-tests.

Results

SAH Physiology

ICP and BP increased and CBF and CPP decreased at SAH (Figure-1). Post-hemorrhage increases in ICP and in BP appeared slightly greater in males than in females but this difference did not reach statistical significance (ICP males: 67 ± 7mmHg, females: 52 ± 4 mmHg; BP rise % baseline males: 123 ± 8 %, females 108± 4 %). CBF fell to similar levels in males and females, while HR remained unchanged at SAH. Taken together these data indicate that the intensity of SAH in male and females was similar and is graded moderate.[8]

Our results are in contrast to our previous findings in 6 month old Wister rats where identical arterial perforation created greater ICP peak and BP rises in the males than in females.[3] We note that the present study used animals of a different age and of a different strain from the previous study and an effect of age and strain on intensity of brain injury is established.[14–16]

Gene Expression

Three different patterns of response are evident among the genes analyzed. A sexually dimorphic response to SAH occurred in Nos3 and Thbd gene expression (Fig. 2). In both cases, expression levels in sham operated animals were similar in the two sexes; however, in both cases SAH caused a 3-5 fold increase in males but no significant change in females. By contrast, Icam1, Il1β and Avp showed significant changes in expression after hemorrhage in both males and females, and the magnitude of *the response was equal in the two sexes* (Fig. 3). Vegf and Tnf-α, showed *no significant response to SAH* and no significant sex difference (Fig. 4).

Discussion

A key finding of this study is that sexual dimorphism in gene expression after SAH exists. Expression of five of the seven genes studied increased in response to SAH, and two of those showed an mRNA response significantly greater in males than in females. It is interesting to note that the products of those genes which exhibited similar responses to SAH in males and females promote inflammation, while those genes which exhibited the sexually dimorphic response inhibit inflammation. Furthermore, the greater expression of anti-inflammatory genes in males functionally counters the known sexually dimorphic pattern of vessel inflammation, which is also greater in males than in females [3]. This pattern could reflect the operation of a feedback loop for which we have no data.

We collected mRNA samples three hours after hemorrhage. At this time, a substantial response to SAH is underway both in the microvasculature and in the brain parenchyma. This response includes adhesion of intravascular neutrophils and platelet aggregates to adluminal endothelial surfaces; entry of substantial numbers of neutrophils and platelets into

brain parenchyma; activation of collagenases and destruction of microvascular basal lamina and of blood-brain barrier, and apoptosis of both neurons and non-neuronal cells.[3, 17]

The sexually dimorphic response

Expression of Nos3 and Thbd genes increased in males and not females after SAH. Nos3 encodes endothelial nitric oxide synthase, a main source of vascular nitric oxide (NO) which is a vasodilator and inhibits platelet aggregation. In male rats, the cerebral NO level falls acutely after SAH and restoration of NO reduces microvessel injury.[18, 19] The effect of SAH on NO level in female rats has not been determined. In humans, Nos3 expression increases early after SAH but it is not currently known if this increase is sexually dimorphic.[20] In contrast, in ischemic stroke patients Nos3 expression has been found to be not sexually dimorphic.[21]

Thbd encodes thrombomodulin (TM), a endothelial membrane surface protein that plays an important role in protecting vasculature, maintaining vessel patency and blood fluidity of vessels.[22] The anti-inflammatory and anti-coagulant effects of thrombomodulin derive from its presence on the adluminal endothelial plasmalemma, and its shedding from the endothelial surface favors thrombosis and associates with vascular disorders. Since circulating soluble TM (sTM) retains only a small fraction of its anti-inflammatory activity, its level is used as a biomarker for endothelial injury and dysfunction.[23] In general, sTM levels are lower in women than in age-matched men.[24, 25] Moreover, in a number of diseases and disorders sexual dimorphism is reported in levels of circulating thrombomodulin. For example, thrombomodulin levels are lower in women with pulmonary thromboembolism or with metastatic carcinoma as compared to their male counterparts.[24, 25] Plasma sTM levels was shown to increase after ischemic stroke, but sexual differences were not addressed.[23] The effect of SAH on cerebral Thbd gene expression has not previously been described. Our data show that Thbd gene expression is upregulated in males following SAH, but not in females.

The sex-equal response

Expression of Icam1, Il1β, and Avp genes increased in both SAH males and females as compared to shams. It is interesting to note that the products of Icam1 and Il1β promote inflammation. *Icam1, a* cell adhesion molecule, aids platelets and neutrophil adhesion to the vessel wall and Il1β, a pro inflammatory cytokine regulates the activity of MMP9, a collagenase implicated in the degradation of vascular basement membrane.[26] Expression of both of these genes has been shown to increase at 24 hours after experimental SAH.[27–29]. The new findings here are that the increase in Icam1 and Il1β gene expression is present substantially earlier, at 3 hours after experimental SAH, and that the response is not sexually dimorphic.

As noted above, we have previously found substantial platelet, neutrophil adhesion to vessel wall and damage to collagen IV lining of basement membrane in both males and females at 3 hours after SAH. The increases we observe in Icam1 and Il1β gene expression in SAH male and female rats could initiate or contribute to those phenomena. Others have shown that Icam1 and Il1β proteins are increased and contribute to both early and delayed brain

injury after SAH.[28] Moreover, neutralization of Icam1 reduces the severity of delayed vasospasm, and neutralization of Il1β reduces activation of peripheral leukocytes after experimental SAH.[30, 31] In SAH patients, high cerebrospinal fluid (CSF) Il1β and blood Icam1 levels associate with poor neurological status.[32, 33] These data suggest that increased expression of these genes after SAH might be a key mechanism driving the post-hemorrhage early inflammatory response.

Avp is a potent vasoconstrictor and contributes to the pathogenesis of vasogenic edema and cellular swelling after stroke and during hyponatremia. We included Avp gene expression in this study since a surge in Avp protein levels occurs at SAH and contributes to the observed rise in BP.[3] The effect of SAH on Avp gene expression has not previously been studied; however, increased in Avp gene expression within hours after ischemic and traumatic brain injury is well established.[6, 7] Hence the increase we observe in Avp gene expression after SAH parallels that in other cerebral injuries. Indirect evidence suggests that the Avp surge at SAH contributes to early brain injury after SAH: inhibition of vasopressin V (1a) receptor prior of SAH blunts BP elevation and reduces the severity of brain damage and mortality in male rats.[34]

Genes unaffected by sex or hemorrhage

No sex- or injury-specific change was present in Vegf and Tnf-α gene expression at 3 hours after SAH. *Vegf,* the protein product of vegf gene is a cytokine involved in increased microvascular permeability. In ischemic stroke Vegf increases in CSF and in plasma and has angiogenic and direct neurotrophic effects.[35] In SAH Vegf gene and protein expression increase at 24 to 72 hours after artery rupture.[4, 5] The lack of change in Vegf gene expression in our study may indicate that this gene upregulates later and not early after SAH.

Tnf-α, the protein product of the Tnf-α gene, is a proinflammatory cytokine that is implicated in endothelial cell apoptosis and BBB permeability after SAH.[36, 37] Increased Tnf-α *protein* has been reported in Wister males at 3 after SAH [38] and in Sprague Dawley males at 7 days after SAH [39] In our Sprague-Dawley rats we found no difference in Tnf-α *gene expression* between sham and SAH operated animals at 3 hours after SAH, and no difference in Tnf-α gene expression between males and females. Clinically, an early increase in cerebral Tnf-α correlates with the size of the ruptured aneurysm and with poor outcome in SAH patients.[40, 41] However, since Tnf-α plays an causative role in cerebral aneurysm formation, the early increase in its level in SAH patients may not be the consequence of aneurysmal rupture but a cause of it.[41] Our data indicate that Tnf-α gene expression does not increase in the first hours after SAH; however, further work is clearly needed in this area.

Our present findings, in agreement with our previous findings, show that the early response to experimental SAH differs between males and females. We find this difference in the extent of vessel pathology and in the expression of genes whose products inhibit inflammation. Our results emphasize the importance of including sex as a variable in experimental studies of SAH and the urgency of considering sex as a factor in current and future clinical studies and in the clinical management of SAH cases.

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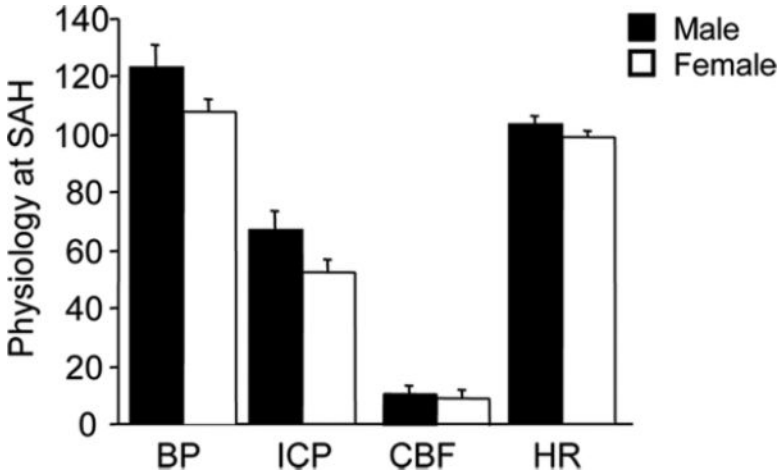

Figure-1. SAH physiology

Intracranial pressure (ICP) and blood pressure (BP) increased, and cerebral blood flow (CBF) fall and heart rate (HR) remained unchanged at SAH induction. The rise in ICP and BP appeared slightly greater in males than in female, but these differences did not reach statistical significance. Data are mean ± sem from 9 male and 9 female animals.

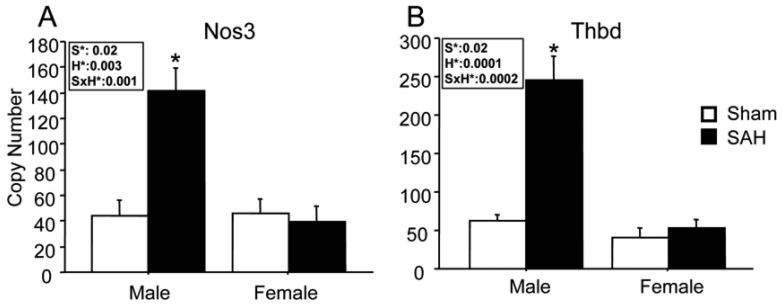

Figure-2. Genes with sexually dimorphic response after SAH

Expression of Nos3 and Thbd genes increased in males and not females after SAH. Open bars: sham; filled bars, SAH. Bars show mean relative mRNA copy number (9 animals per group). Inset boxes show ANOVA effect of sex (S), hemorrhage (H) and interaction between the two (SxH) for each gene. Effects which reach significance are in bold. 2 way ANOVA results**: A; Nos3**: sex effect (S): F=6, p=0.02; hemorrhage effect (H): F=11, p=0.003; sex by hemorrhage interaction (S*H): F=15, p=0.001. **B; Thbd**: S: F=7, p=0.02; H: F; 30, p<0.0001; S*H: F=23, p=0.0002. Data are mean ± sem. * significantly difference at P<0.05 in comparison of hemorrhage vs sham within sex.

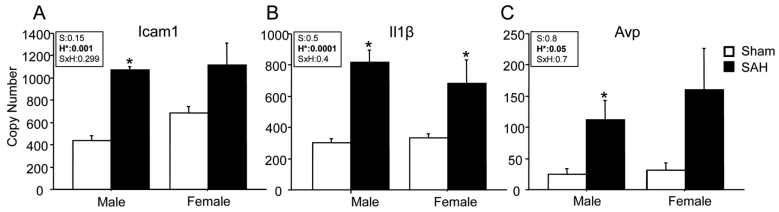

Figure-3. Genes with sex-equal response

Expression of Icam1, Il1β and Avp genes increased in both SAH males and females as compared to shams. Open bars: sham; filled bars, SAH. Bars show mean relative mRNA copy number (9 animals per group). Inset boxes show ANOVA effect of sex (S), hemorrhage (H) and interaction between the two (SxH) for each gene. Effects which reach significance are in bold. 2 way ANOVA results**: A; Icam1**: sex effect (S): F=2, p=0. 2; hemorrhage effect (H): F=24, p=0.0001; sex by hemorrhage interaction (S*H): F=1.2, p=0.3. **B; Il1β**: S: F=0.6, p=0.6; H: F=25, p=0.0001; S*H: F=1, p=0.3. **C; Avp**: S: F=0.05, p=0.8; H: F-5, p=0.045; S*H: F=0.2, p=0.7. Data are mean ± sem. *significantly difference at P<0.05 in comparison of hemorrhage vs sham within sex.

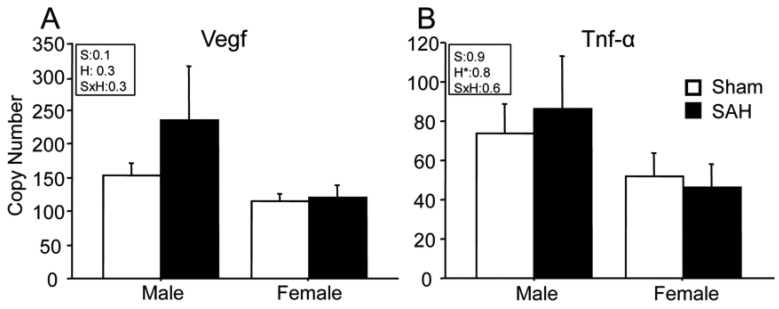

Figure-4. Genes unaffected by sex or hemorrhage

No sex -or injury- specific differences were present in Vegf and Tnf-α gene expression at 3 hours after SAH. Bars show mean relative mRNA copy number (9 animals per group). Inset boxes show ANOVA effect of sex (S), hemorrhage (H) and interaction between the two (SxH) for each gene. Effects which reach significance are in bold. 2 way ANOVA results**: A; Vegf**: sex effect (S): F=0.1, p=0.7; hemorrhage effect (H): F=1, p=0.3; sex by hemorrhage interaction (S*H): F=1, p=0.3. **B; Tnf-α**: S: F=0.007, p=0.9; H: F=0.05, p=0.8; S*H: F=0.3, p=0.6. Data are mean ± sem. *significantly difference at P<0.05 in comparison of hemorrhage vs sham within sex.

Table 1

Accession numbers and primer sequences

Gene Name	Abbreviation	Accession Number	Primer sequence
Intracellular adhesion molecule-1	Icam1	NM_012967.1	*S:* TGATATCCGGTAGACACAAGCA *AS:* CTTCACCAGGGTCTAGGCAA
Endothelial nitric oxide synthase	eNos or Nos3	NM_021838.2	*S:* TGAAGCCGACGCTCATGCACA *AS:* ATTGCCTCGGTTTGTTGCAT
Thrombomodulin	Thbd	NM_031771.2	*S:* ACTCTCCACTGTGTTTGCCAT AS: GACTCCTTTCCCAAGTTGCCAT
Vascular endothelial growth factor	Vegf	NM_001287107.1	S: GCTCACTTCCAGAAACACGAC *AS:* ATCCACAATAGTGCCATGTCC
Interleukin-1beta	Il1β	NM_031512.2	*S:* TACATCAGCACCTCTCAAGCAG *AS:* AAGTCAACTATGTCCCGACCA
Tumor necrosis factor-alpha	Tnf-α	NM_012675.3	*S:*CAGTTCCATGGCCCAGACCCTC *AS:* ACTCCAGCTGCTCCTCCGCTTG
Arginine vasopressin	Avp	NM_016992.2	*S:* ACCTCTGCCTGCTACTTCCA *AS:* GTCTCAGCTCCATGTCGGAT

Primers were designed using BLAST program and were purchased from Eurofins MWG Operon. S: sense, AS antisense.