A potential role for inducible nitric oxide synthase in the cerebral response to acute hemodilution

Le rôle potentiel de la monoxyde d'azote synthase inductible dans la réponse cérébrale à l'hémodilution aiguë

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Abstract

Purpose Acute anemia increases the cerebral expression of hypoxic molecules including neuronal nitric oxide synthase (nNOS) and hypoxia inducible factor-1 α (HIF-1 α). This study assessed the effects of acute hemodilution on inducible NOS (iNOS) and systemic inflammatory cytokines.

Methods Anesthetized rats ($n = 5-7$ per group) underwent 50% hemodilution with pentastarch, whole blood exchange or no fluid exchange. Cerebral cortical nNOS and iNOS protein levels were characterized using Western blot analysis and immunostaining (1 and 18 h). Plasma cytokine levels were assessed by enzyme-linked immunosorbent assay (1, 4, and 18 h). Data were analyzed by two-way analysis of variance to determine significance ($P < 0.05$, mean $\pm$ SD).

Results No differences in mean arterial blood pressure or arterial blood gases were observed between groups

after hemodilution. A comparable hemoglobin target ($\sim 70 \text{ g} \cdot \text{L}^{-1}$) was achieved in all groups following hemodilution. Cerebral cortical iNOS protein levels were increased in anemic rats, relative to controls. The nNOS protein levels increased to a greater degree ($P < 0.05$ for both). Immunostaining demonstrated that iNOS localized to endothelium, glial fibrillary acidic protein (GFAP) positive (astrocytes) and GFAP negative cells within the brain. Plasma cytokine levels (tumour necrosis factor α , interleukin (IL)-1 β and IL-6) increased transiently, to the same levels, in both control and hemodiluted rats.

Conclusions Cerebral cortical iNOS and nNOS protein levels were both increased in anemic rats. The nNOS response was predominant. This suggests that NOS-derived NO may be an important signalling pathway which is activated in the brain during anemia. These cellular responses could maintain cerebral homeostasis, or contribute to neuronal injury, during acute hemodilutional anemia.

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Résumé

Objectif L'anémie aiguë augmente l'expression cérébrale des molécules de l'hypoxie, notamment de la monoxyde d'azote synthase neuronale (nNOS) et du facteur-1 α induit par l'hypoxie (HIF-1 α). Cette étude a évalué les effets d'une hémodilution aiguë sur le NOS inductible (iNOS) et les cytokines inflammatoires systémiques.

Méthode Des rats anesthésiés ($n = 5-7$ par groupe) ont subi une hémodilution à 50 % avec du pentastarch, un échange de sang total ou aucun échange liquidien. Les niveaux de protéines nNOS et iNOS dans le cortex cérébral ont été caractérisées à l'aide de tests de buvardage de western et d'immunocoloration (1 et 18 heures). Les niveaux de cytokines plasmatiques ont été évalués par un test ELISA (1, 4 et 18 heures). Les données ont été

analysées par une analyse de la variance à deux critères de classification afin de déterminer leur pertinence ($P < 0,05$, moyenne $\pm$ ET).

Résultats Aucune différence n'a été observée dans la pression artérielle moyenne et les gaz du sang entre les groupes après l'hémodilution. Une cible d'hémoglobine comparable ($\sim 70 \text{ g}\cdot\text{L}^{-1}$) a été obtenue dans tous les groupes après l'hémodilution. Les niveaux de protéines iNOS dans le cortex cérébral étaient plus élevés chez les rats anémiques par rapport au groupe témoin. Les niveaux de protéines nNOS ont davantage augmenté ($P < 0,05$ dans les deux groupes). L'immunocoloration a démontré que les iNOS étaient situés dans l'endothélium, sur les cellules d'acide protéique fibrillaire glial (GFAP) positives (astrocytes) et sur les cellules GFAP négatives dans le cerveau. Les niveaux de cytokines plasmatiques (facteur onconécrosant- α , interleukine (IL)-1 β et IL-6) ont temporairement augmenté, aux mêmes niveaux dans le groupe témoin et chez les rats hémodilués.

Conclusion Les niveaux de protéines iNOS et nNOS cortico-cérébrales ont tous deux augmenté chez les rats anémiques. La réponse nNOS était prédominante, ce qui suggère que le NO dérivé de NOS pourrait constituer une voie de signalisation importante activée dans le cerveau pendant l'anémie. Ces réponses cellulaires pourraient maintenir l'homéostasie cérébrale, ou contribuer à des lésions neuronales, pendant une anémie aiguë induite par l'hémodilution.

Acute anemia contributes to morbidity and mortality in surgical patients.¹⁻⁴ In this setting, acute anemia results from acute hemorrhage (*blood loss*), fluid resuscitation (*hemodilution*), or a combination of both. Cumulative evidence from clinical studies suggests that anemia-induced injury to vital organs, including the brain^{3,5-8} and kidney,^{3,8-10} may contribute to the observed increase in mortality. In addition, the main treatment strategy for acute blood loss (*allogeneic blood transfusion*) is associated with increased mortality.¹¹⁻¹⁵ A clearer understanding of the cellular responses during acute anemia is required to determine the potential mechanisms of injury.

We have previously demonstrated that hypoxia inducible factor-1 α (HIF-1 α) and neuronal nitric oxide synthase (nNOS) protein levels are increased in the brain of anemic rats. It is not yet clear whether these cellular responses are adaptive or maladaptive.^{16,17} By contrast, endothelial nitric oxide synthase (eNOS) messenger ribonucleic acid (mRNA) and protein levels were not shown to be upregulated in our model.^{16,17} We also demonstrated that increased HIF levels can lead to upregulation of inducible

nitric oxide synthase (iNOS) mRNA.¹⁷ However, experimental studies have not yet demonstrated whether iNOS protein levels are also increased in the brain during acute anemia. iNOS has been shown to contribute to both adaptive (*ischemic Pre-conditioning*) and maladaptive (*inflammatory*) mechanisms within the brain.^{18,19} Therefore, measuring cerebral iNOS protein levels after acute hemodilution may help to determine if this NOS isoform is important during acute anemia. In addition, we wished to determine if increased inflammatory cytokine could contribute. We proposed to test the null hypothesis that acute hemodilution does not result in an increase in cerebral cortical iNOS protein levels.

Methods

Animal model

All animal protocols were approved by the Animal Care Committee at St. Michael's Hospital. Male Sprague-Dawley rats were anesthetized with 2% isoflurane (Abbott, St. Laurent, QC, Canada) in 50% oxygen and their lungs were ventilated to achieve normocapnia and normoxia. The tail artery and vein were cannulated (Angiocath 24G) to measure mean arterial blood pressure, arterial blood gases (ABGs), and hemoglobin concentration by co-oximetry (Radiometer ALB 500 and OSM 3, London Scientific, London, ON, Canada), and to perform acute hemodilution. Rectal temperature was maintained near 37°C, and all physiological data were acquired digitally (Power Lab 16/30, ADInstruments, Colorado Springs, CO, USA).

Fig. 1 Experimental time lines

Hemodilution protocol

Two experimental protocols were performed (Fig. 1). *Protocol #1*: Control rats underwent acute volume exchange (50% of the estimated blood volume, $30 \text{ mL} \cdot \text{kg}^{-1}$) with strain-specific whole blood to control for the hemodynamic effects of fluid exchange. Rats in the experimental anemia group underwent 50% hemodilution by simultaneously exchanging an equal volume of blood ($30 \text{ mL} \cdot \text{kg}^{-1}$) with pentastarch over 10 min. Two different groups of control and anemic rats were then recovered for either 1 or 18 h ($n = 5\text{--}7$ rats per group). Fresh whole blood for re-infusion was obtained from isoflurane-anesthetized rats by aortic puncture ($n = 11$). An additional six rats underwent hemodilution for immunohistochemistry. *Protocol #2*: In the second experiment, plasma cytokines were measured in anemic rats in which 50% hemodilution was performed with pentastarch, and they were also measured in control rats that did not undergo hemodilution or blood exchange (anesthesia-ventilation and surgery controls). Blood exchange was not performed in this group of control rats in order to avoid possible changes in inflammatory mediators associated with whole blood exchange. In addition to ABG and co-oximetry measurements, these rats had 1 mL of blood sampled for cytokine measurements at baseline, as well as 1 and 4 h following hemodilution. The rats were then recovered for 18 h and then re-anesthetized, and a final blood sample was taken to determine cytokine levels (Fig. 1).

Western blot, immunohistochemistry

Western analysis and immunostaining were performed using specific primary antibodies as previously reported.¹⁷ Briefly, total protein was extracted from cerebral cortical tissue and total protein concentration measured (*Lowry method*). Protein samples ($50 \mu\text{m}$ per well) underwent electrophoresis (SDS-PAGE) under reducing conditions and were then transferred to a nitrocellulose membrane. Specific protein bands were detected using specific primary (iNOS #610431, nNOS #610309, eNOS #610296 BD Biosciences, β actin; A5441, Sigma) and appropriate secondary antibodies. Protein band densities were quantified digitally and normalized to the loading control (β actin). *Immunohistochemical* staining was performed on $10 \mu\text{m}$ fixed tissue sections (4% paraformaldehyde) by incubating slides overnight at 4°C with diluents of specific primary monoclonal antibodies (HIF-1 α ; NB 100-105, Novus Biologicals, iNOS #610431, BD Transduction, glial fibrillary acidic protein (GFAP)-Cy3, C9205 Sigma, To-Pro-3 #T3605 Invitrogen Mol Probes), and specific binding was detected with an appropriately labelled secondary antibody.¹⁷ Microscopy was performed utilizing standard and

confocal microscopes (Nikon ECLIPSE 90i, Bio-Rad Radiance 2100).

Cytokine measurement by ELISA

To obtain plasma, blood samples were centrifuged at $5000g$ at 4°C for 10 min immediately after collection. The supernatant was removed and samples aliquoted into microfuge tubes on ice before being snap frozen at -80°C . Cytokines were assayed using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA). Samples were thawed at room temperature before being added to microtiter strips precoated for tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , or IL-6 antigens (Cat # KRC3012, KRC0012, and KRC0062, respectively, Mediacorp Incorporated, Montreal, QC, Canada). All samples were run in duplicate. After the reaction was stopped, microtiter plates were analyzed at 450 nm by a microtiter plate reader. The mean absorbance was taken, and cytokine concentrations were calculated using specific standard curves.

Data analysis

For experimental outcomes in *Protocol #1*, ABG, co-oximetry data, and cerebral NOS protein levels were assessed in control and hemodiluted rats at 1 and 18 h by a two-way analysis of variance (ANOVA) to assess any significant time or group effects. In all cases, a post hoc Tukey test was performed for specific comparisons if a significant F ratio was achieved. For *Protocol #2*, a two-way repeated measure ANOVA was utilized, since four sequential blood samples were taken from each rat. Each cytokine was assessed individually. No adjustment was made for the entire experiment-wise error in this study. All data are expressed as mean $\pm$ standard deviation and significance assigned at a value of $P < 0.05$.

Results

Arterial blood gas and co-oximetry data

No differences in ABG values were identified between groups after hemodilution (Tables 1 and 2). Target hemoglobin levels decreased to $60\text{--}70 \text{ g} \cdot \text{L}^{-1}$ in all hemodiluted rats. There was a slight increase in methemoglobin levels in hemodiluted rats relative to controls (Tables 1 and 2).

Cerebral cortical nNOS, iNOS, and eNOS protein levels following acute hemodilution

Cerebral cortical nNOS protein levels were not increased at 1 h after hemodilution. After 18 h, nNOS protein levels in

Table 1 Arterial blood gas and co-oximetry data following blood or pentastarch exchange

Recovery time	Sample time (min)	Description	pH	P _a CO ₂ (mmHg)	P _a O ₂ (mmHg)	Hg (g · L ⁻¹)	% Saturation	MetHb (%)	O ₂ content (mmol · L ⁻¹)
<i>Control-blood exchange, 50% (n = 5–6)</i>									
1 h	10	Baseline	7.46 ± 0.09	36.7 ± 8.3	166.3 ± 49.6	129 ± 3	99.8 ± 0.6	1.2 ± 0.1	7.7 ± 0.2
1 h	70	Post exchange	7.41 ± 0.08	39.8 ± 8.7	188.0 ± 36.6	122 ± 8	99.9 ± 0.1	1.2 ± 0.1	7.3 ± 0.5
18 h	10	Baseline	7.44 ± 0.08	37.3 ± 6.8	155.4 ± 40.0	123 ± 7	99.6 ± 0.7	1.3 ± 0.1	7.4 ± 0.4
18 h	70	Post exchange	7.43 ± 0.05	34.8 ± 5.5	166.7 ± 34.5	118 ± 6	99.8 ± 0.5	1.3 ± 0.1	7.0 ± 0.3
<i>Anemia-pentastarch exchange, 50% (n = 6–7)</i>									
1 h	10	Baseline	7.35 ± 0.13	40.4 ± 5.5	167.3 ± 22.3	125 ± 10	99.9 ± 0.1	1.1 ± 0.1	7.5 ± 0.6
1 h	70	Post exchange	7.32 ± 0.08	42.2 ± 8.1	191.5 ± 19.2	63 ± 9*	99.9 ± 0.1	1.5 ± 0.2*	3.8 ± 0.5*
18 h	10	Baseline	7.40 ± 0.02	39.0 ± 4.2	164.0 ± 29.5	119 ± 8	99.9 ± 0.1	1.1 ± 0.2	7.2 ± 0.6
18 h	70	Post exchange	7.40 ± 0.06	40.3 ± 7.4	187.8 ± 35.1	59 ± 3* [#]	100.0 ± 0.1	1.6 ± 0.2* [#]	3.5 ± 0.2* [#]

Post hoc Tukeys, * $P < 0.05$, relative to baseline (10 min), [#] $P < 0.05$, relative to control

Table 2 Arterial blood gas and co-oximetry data following control or pentastarch hemodilution (recovery experiment)

Time (min)	Description	pH	P _a CO ₂ (mmHg)	P _a O ₂ (mmHg)	Hg (g · L ⁻¹)	% Saturation	MetHb (%)	O ₂ content (mmol · L ⁻¹)
<i>Control—no volume exchange (n = 6)</i>								
10	Baseline	7.43 ± 0.05	38.0 ± 6.3	190.5 ± 22.6	133 ± 10	99.9 ± 0.1	1.3 ± 0.2	7.9 ± 0.6
70	Post 1	7.39 ± 0.05	42.3 ± 5.9	192.2 ± 26.9	124 ± 1	100.0 ± 0.1	1.3 ± 0.1	7.4 ± 0.4
320	Post 2	7.42 ± 0.04	38.3 ± 6.8	209.3 ± 49.7	110 ± 6*	99.7 ± 0.9	1.5 ± 0.1	6.5 ± 0.4*
<i>Anemia—pentastarch exchange, 50% (n = 6)</i>								
10	Baseline	7.40 ± 0.02	39.0 ± 4.2	164.0 ± 29.5	132 ± 5	99.9 ± 0.1	1.3 ± 0.1	7.9 ± 0.4
70	Post exchange 1	7.40 ± 0.06	40.3 ± 7.4	187.8 ± 35.1	63 ± 3* [#]	100.0 ± 0.1	1.7 ± 0.2* [#]	3.7 ± 0.2* [#]
320	Post exchange 2	7.43 ± 0.05	34.8 ± 5.5	166.7 ± 34.5	70 ± 6* [#]	100.0 ± 0.1	1.8 ± 0.2* [#]	4.2 ± 0.4* [#]

Post hoc Tukeys, * $P < 0.05$, relative to baseline (10 min), [#] $P < 0.05$, relative to control

Table 3 Comparison of normalized neuronal, inducible, and endothelial nitric oxide synthase protein levels after 1 and 18 h of anemia

Normalized NOS protein levels (n = 5–7)					
Group	NOS isoform	Protein level		ANOVA— P value	
		1 h	18 h	Time	Group
Control (C)	nNOS	1.00 ± 0.32	1.00 ± 0.30	0.008	0.026
Anemia (A)	nNOS	0.93 ± 0.16	1.67 ± 0.39***		
Control (C)	iNOS	1.00 ± 0.23	1.00 ± 0.24	0.653	0.020
Anemia (A)	iNOS	1.27 ± 0.49***	1.39 ± 0.15***		
Control (C)	eNOS	1.00 ± 0.20	1.00 ± 0.21	0.177	0.110
Anemia (A)	eNOS	0.81 ± 0.03	0.98 ± 0.08		

NOS nitric oxide synthase, ANOVA analysis of variance; nNOS neuronal nitric oxide synthase; iNOS inducible nitric oxide synthase; eNOS endothelial nitric oxide synthase

ANOVA *** $P = 0.020$. Post hoc Tukeys analysis * $P < 0.05$, relative to anemia 1 h; ** $P < 0.05$, relative to control 18 h

hemodiluted rats had increased by 60–100% relative to controls ($P < 0.05$, Table 3, Fig. 2). Cerebral cortical iNOS protein levels increased by approximately 30% at both 1 and 18 h following hemodilution (Group effect, $P = 0.020$, Table 3, Fig. 3). No increase in eNOS protein levels was observed (Table 3, Fig. 3).

Immunohistochemistry demonstrated that iNOS localized to the endothelium, to GFAP positive astrocytes, and to GFAP negative cells, which resembled neurons in the brain of hemodiluted rats (Fig. 4). Brain sections from anemic rats also demonstrated that HIF-1 α staining was increased in astrocytes that were associated with cerebral

Fig. 2 Relative to controls, which underwent whole blood exchange (open symbols), after acute hemodilution (black symbols), cerebral cortical neuronal nitric oxide synthase (nNOS) are increased at 18 h (black triangles) but not at 1 h (black circles)

Cerebral Cortical nNOS, eNOS and iNOS Protein

Fig. 3 Both neuronal (nNOS) and inducible (iNOS) nitric oxide synthase protein levels are increased in the cerebral cortex of anemic rats at 18 h, while endothelial NOS (eNOS) is not increased

blood vessels (Fig. 5). In addition, nNOS positive neurons were located near astrocytes and cerebral blood vessels in the anemic cerebral cortex.

Cytokines responses to anesthesia and hemodilution

Plasma IL-1 β levels increased in both groups over the first 4 h following hemodilution and reached peak levels that were twofold higher than baseline in both groups (22.6 ± 5.4 and 21.6 ± 15.5 pg $\cdot$ mL $^{-1}$; $P < 0.05$ relative to baseline, Fig. 6). IL-1 β levels then returned to baseline by 18 h. Plasma TNF α levels increased dramatically by 5000-fold in both groups at 1 h (5634 ± 1507 vs

Fig. 4 Immunohistochemistry demonstrates inducible nitric oxide synthase (iNOS) positive staining (green) in endothelium (a), glial fibrillary acidic protein (GFAP) positive astrocytes (red) (b), and GFAP negative cells that resemble neurons (c) in brain from hemodiluted rats (18 h). Proximity of stained cells to cerebral blood vessels is indicated by white arrows

HIF-1 α and nNOS (Green) Localize to Astrocytes (Red) and Neurons in the Brain of Anemic Rats

Fig. 5 Immunohistochemistry demonstrated perivascular localization of glial fibrillary acidic protein (GFAP) (red) positive astrocytes that stained positively for hypoxia inducible factor-1 α (HIF-1 α , green) in the cerebral cortex of anemic rats (a). Increased neuronal nitric oxide synthase (nNOS, green) was identified within perivascular neurons that did not stain for the astrocytic marker GFAP (red) (b). Proximity of stained cells to cerebral blood vessels is indicated by white arrows

5801 ± 2058 pg $\cdot$ mL $^{-1}$; $P < 0.05$ relative to baseline, Fig. 6) before rapidly returning to baseline at 4 h. Plasma IL-6 levels were not detected in baseline samples but increased comparably in both groups at 1 h (496 ± 290 vs 575 ± 291 pg $\cdot$ mL $^{-1}$) before returning to undetectable levels at 18 h.

Fig. 6 Transient increases in inflammatory cytokines interleukin-1 β (IL-1 β) and tumour necrosis factor α (TNF α) are comparable in hemodiluted (black circles) and control rats (white circles)

Discussion

This experimental study demonstrated that both iNOS and nNOS protein levels increased in the cerebral cortex of anemic rats following acute hemodilution. This is consistent with our previous research, which has demonstrated increases in both iNOS and nNOS mRNA levels in anemic rats. This suggests that anemia-induced regulation of NOS occurs at the transcriptional level. Increased iNOS expression may be mediated by HIF-1 α , which promotes transcription of iNOS.^{16,17} However, plasma cytokines did not appear to contribute to the observed increase in cerebral iNOS expression. Cellular localization by immunofluorescence suggests that iNOS expression was increased in endothelial cells, perivascular astrocytes, and neurons in the anemic rat brain. Thus, iNOS derived NO may play an important role in cell signalling events within the brain of acutely anemic rats. The relative increase in nNOS protein exceeded the iNOS response, and there was no increase in eNOS protein. Further experimental studies are required to assess the relative roles of iNOS- and nNOS-derived NO within the cerebral cortex of anemic mammals.

Two clinical studies have reported an increase in patient mortality when treatment strategies reducing the biological availability of systemic NO were utilized in acutely ill patients.^{20,21} Therefore, NO signalling may be important for survival under conditions of hemodynamic stress. The increase in cerebral NOS expression during acute anemia may play an important adaptive role. Further studies are required to characterize the functional roles of nNOS and iNOS during acute anemia.

Conceptually, hemodilution could activate cerebral NOS and other adaptive mechanisms by three potential pathways: (1) a direct vascular signalling event mediated via the vascular endothelium and directed toward the immediately adjacent vascular smooth muscle and perivascular cells (*astrocytes and neurons*); (2) changes in the local cerebral tissue environment (*hypoxia*) secondary to reduced cerebral oxygen delivery; and (3) changes in remote tissue environments (*hypoxia*) sensed by local chemoreceptors that trigger afferent neural responses to the central nervous system (Fig. 7). Evidence supports the activation of NOS by each of these pathways. First, evidence of increased iNOS expression in the endothelium suggests that a vascular signal event may have contributed to the increase in cerebral cortical iNOS expression in anemic rats. Second, although we and others have been unable to demonstrate cerebral tissue hypoxia at the hemoglobin levels achieved in this study, changes in the local cerebral environment, including hypoxic gene expression, have been documented.¹⁶ Despite the lack of cerebral tissue hypoxia, HIF-1 α (*hypoxic transcription factor*) protein levels increased the cerebral cortex of anemic rats.²² This paradoxical increase in HIF-1 α stabilization may be mediated by NOS-derived NO under normoxic tissue conditions as previously hypothesized.²² Stabilized HIF-1 α may then upregulate iNOS in a positive feed-forward manner. Further experimental studies are required to characterize these mechanisms. Finally, hemodilution to hemoglobin levels achieved in his study (70 g $\cdot$ L⁻¹) have been associated with intestinal and renal tissue hypoxia.^{23,24} Thus, anemia-induced peripheral tissue hypoxia (*kidney, intestine*) may trigger an afferent neuronal signal to the brain. Central processing of this signal could initiate an appropriate efferent neuronal response that may stimulate perivascular nerves to increasing nNOS derived NO production and thereby promote cerebral vasodilation²⁵ (Fig. 7). Such NOS–NO signalling events may be critical to optimizing cerebral perfusion and maintaining cellular homeostasis during acute anemia. The characterization of these pathways may help to reduce morbidity and mortality associated with acute anemia.

Cytokines, including TNF α and IL-1 β , have been demonstrated to upregulate HIF and iNOS expression.^{26–28} Therefore, we speculated that changes in plasma cytokines

Fig. 7 Schematic representation illustrating potential nitric oxide synthase (NOS) signalling pathways within perivascular cerebral tissue, including neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) isoforms, which may be activated during acute hemodilution. Hemodilutional anemia causes tissue hypoxia in specific organs (i.e., kidney) which could send afferent neuronal signals to the central nervous system (CNS) thereby activating appropriate efferent responses, including an increase in nNOS derived nitric oxide (NO) from perivascular neurons or vascular smooth muscle (VSM). Alternately, hemodilution may activate receptors within the vascular luminal, which results in upregulation of perivascular NOS derived NO

might provide the stimulus for increased HIF-1 α and iNOS observed within the cerebral cortex of anemic rats. Our data did not support this finding. Interestingly, there was a dramatic increase in TNF α , IL-1 β , and IL-6 in control and anemic animals. This suggests that stimuli, such as anesthesia, surgery, and mechanical ventilation, provide a strong stimulus to increase systemic cytokine levels. However, these increases were transient and occurred in both the anemic and the control groups. Therefore, these cytokines are not likely to be responsible for the observed increase in nNOS, iNOS, and HIF protein levels observed within the cerebral cortex of anemic mice.

There are some limitations to our study. Cerebral cortical iNOS protein levels did not increase over time and were only mildly elevated ($\sim 30\%$). By contrast, nNOS increased to a larger degree over time. However, both types of signals (*small and sustained vs large and transient*) may contribute to physiological regulation of biological mechanisms. The finding that both iNOS and nNOS mRNA levels are increased during anemia supports a transcriptional mechanism of regulation for both proteins.^{16,17} In addition, we did not measure NOS activity or NO metabolite levels.

In conclusion, the current study suggests that acute hemodilution leads to an increase in protein levels of two NOS isoforms, nNOS and iNOS. This suggests that NOS derived NO may have important signalling roles that could

maintain cerebral homeostasis during acute anemia. Alternately, NOS derived NO may be maladaptive and may contribute to anemia-induced cerebral injury.

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Conflicts of interest None declared.

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