Acute Anemia Elicits Cognitive Dysfunction and Evidence of Cerebral Cellular Hypoxia in Older Rats with Systemic Hypertension

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ABSTRACT

Background: Postoperative cognitive dysfunction occurs frequently after cardiac, major vascular, and major orthopedic surgery. Aging and hypertensive cerebrovascular disease are leading risk factors for this disorder. Acute anemia, common to major surgery, has been identified as a possible contributor to postoperative cognitive dysfunction. The effect of hypoxia upon cognition and the cellular and molecular processes involved in learning and memory has been well described. Cerebrovascular changes related to chronic hypertension may expose cells to increased hypoxia with anemia.

Methods: Young to aged spontaneously hypertensive rats underwent testing for visuospatial memory and learning in the Morris water maze, measurement of cerebral tissue oxygenation *via* tissue oxygen probe, and measurement of hypoxia-sensitive genes and proteins, under conditions of sham and experimental isovolemic anemia.

Results: Acute isovolemic anemia elicited evidence of aging-dependent visuospatial working memory and learning impairment. Isovolemic anemia did not result in cerebral tissue hypoxia, when measured *via* tissue oxygen probe. Evidence of cellular hypoxia was, however, identified in response to the anemic challenge, as hypoxia-sensitive genes and proteins were up-regulated. Importantly, cellular hypoxic gene responses were increased with anemia in an age-dependent manner in this model of aging with chronic hypertension.

Conclusions: In a translational model of chronic hypertension, clinically relevant levels of acute anemia were associated with an age-dependent visuospatial working memory and learning impairment that was matched by an age-dependent cellular sensitivity to anemic hypoxia. These data offer support for a possible link between anemic hypoxia and postoperative cognitive dysfunction in humans.

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What We Already Know about This Topic

❖ Postoperative cognitive dysfunction occurs more commonly in the aged. Anemia has been associated with postoperative cognitive impairment.

What This Article Tells Us That Is New

❖ In a laboratory study of young *versus* aged spontaneously hypertensive rats, acute isovolemic anemia produced age-dependent impairment of learning and memory as well as molecular signs of cellular hypoxia.

❖ These animal data suggest a possible link among anemia, cellular hypoxia, and postoperative cognitive dysfunction with aging.

POSTOPERATIVE cognitive dysfunction (POCD) primarily affects the working memory domains of attention, cognitive speed, and executive function, as well as hippocampal based memory acquisition or learning.[1]

⊕ Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of the article on the Journal's Web site (www.anesthesiology.org).

POCD may occur in 40–60% of patients after cardiac surgery and somewhat less frequently after major vascular and orthopedic surgery.[1,2] Aging[3] appears to be the most important risk factor for its occurrence. Patients presenting for cardiac and noncardiac surgery often suffer from longstanding hypertension and preexisting hypertension-related cerebrovascular disease, also an important risk factor for cognitive impairment.[4]

Mechanisms potentially contributing to POCD remain poorly understood. Research has focused largely on processes related to the conduct of cardiopulmonary bypass in cardiac surgery. The "Off-Pump" approach for coronary artery bypass grafting, designed to avoid the presumed cerebral side effects associated with extracorporeal circulation, has failed to diminish the incidence of POCD.[5] In light of this, and the parallel occurrence of POCD after major noncardiac surgery, it is plausible that POCD after both major cardiac and noncardiac surgery shares common origins, at least in part unrelated to anesthetic exposure. Finally, although POCD occurs with high frequency postoperatively, it resolves in most cases over several weeks to months, and there is little evidence of permanent disability.[6] Is there then a contributing process that shares such a time course?

We propose that a role for anemic hypoxia be considered in the genesis of POCD. Acute anemia has been found by Weiskopf et al.[7] to elicit cognitive impairment even in healthy nonsurgical subjects. Major surgery in all arenas frequently results in acute and severe anemia. Severe anemia during cardiopulmonary bypass has been shown to lead to developmental delay in infants after cardiac surgery.[8] Kulier et al.[9] and Karkouti et al.[10] have demonstrated a correlation between adverse cerebral outcomes and lower preoperative hemoglobin levels. Mathew et al.[11] randomized 108 elderly subjects to hematocrits of more than 27% or less than 18% during cardiopulmonary bypass, with subjects undergoing more profound hemodilution experiencing a greater degree of cognitive impairment. Finally, anemia is recoverable over a time course similar to recovery from POCD.[12]

Mild to moderate hypoxia associated with high-altitude exposure and intermittent hypoxia associated with sleep apnea have also been linked to working memory and learning impairment.[13–15] It is not surprising then that mild to moderate hypoxia has also been found to impair neuronal protein synthesis and synaptic plasticity,[16] key processes requisite for learning. Exposure to hypoxia triggers a cascade of events focused upon cell survival. The hypoxia-inducible factor (HIF) transcription system[17] is the "master" regulator of this hypoxic response.[18] The prolyl hydroxylases family of proteins (prolyl hydroxylases-1, -2, and -3), along with factor-inhibiting HIF, responsible for degrading ubiquitously present HIF during normoxia, are themselves inhibited during hypoxia, allowing levels of HIF to rise.[19] This results in the transcription of hundreds of hypoxia-responsive genes[20] involved in regulating a host of activities focused on cell survival, including glucose transport (glucose transporter-1, GLUT-1), glycolysis (phosphoglycerate kinase-1, PGK-1),

oxygen transport (erythropoiesis-erythropoeitin), and angiogenesis (vascular endothelial growth factor, VGEF).[21] Effective hypoxia sensing and adaptation to hypoxia are critical for cellular function in all organs, including the brain.[22] HIF and related gene products are exquisitely sensitive biomarkers of cellular hypoxia. Critical to the management of our aging population, aging cells appear to have a diminished, putatively cytoprotective, hypoxic response.[23]

In this study, we test the hypothesis that acute isovolemic anemia leads to cognitive impairment with aging. We also test the hypothesis that aging with hypertension predisposes to cerebral tissue and cellular hypoxia with anemia. These experiments were conducted in a rodent model of chronic hypertension and cerebrovascular disease, the spontaneously hypertensive rat (SHR). We chose the SHR rat model in which to test these hypotheses because of the many similarities between this strain and humans with hypertensive cerebrovascular disease who present for cardiovascular, vascular, and major noncardiovascular surgery.

Materials and Methods

Methodologies are described below for the three main experiments conducted: 1) memory testing with anemia, 2) cerebral oxygenation with anemia, and 3) molecular markers of hypoxia with anemia.

Approvals

Approval from the Institutional Animal Care and Use Committee at the University of Pennsylvania, Philadelphia, Pennsylvania, was obtained before the conduct of this study.

Animals and Experimental Groups

Experiments were conducted in a rodent model of chronic hypertension and cerebrovascular disease, the spontaneously hypertensive rat. In this model, hypertension starts developing by 4 months of age with the appearance of hypertension-related changes in the brain microvasculature by 6 months of age.[24] Mortality in the SHR is approximately 50% at 18 months of age.[25] SHR were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN). A more detailed description of cerebrovascular changes in this model can be found in Supplemental Digital Content 1, which provides a more detailed description of methods for all experiments than can be provided in the main manuscript, http://links.lww.com/ALN/A613. Three age groups were studied: young = 3 months (Y), mature = 9–12 months (M), and aged = 13–18 months (A). Two treatment subgroups were included. The anemic (An) subgroups, YAn, MAn, and AAn, underwent the true hemodilution protocol, and a sham (Sh) protocol was conducted in the YSh, MSh, and ASh subgroups.

Anesthetic Management

To conduct the hemodilution and sham hemodilution protocols for the memory testing and molecular markers of hypoxia experiments, rats were anesthetized in a plastic box con-
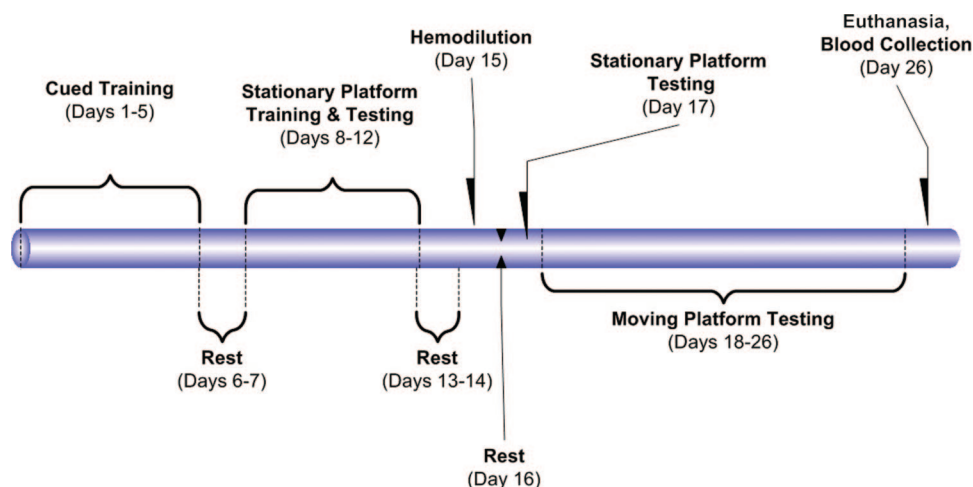
Fig. 1. Training and testing timeline. Five days of cued (flagged) training on a fixed, submerged platform (days 1–5) was followed by 2 days of rest (days 6–7). Noncued training (days 8–12) occurred on the same fixed platform location, with visual cues as to platform location now available from the surrounding room only. Two days of rest followed (days 13 and 14). Hemodilution or sham hemodilution (day 15) was followed by 1 day of rest (day 16). Reference memory testing (day 17) examined the ability to remember the position of the platform trained upon during the prehemodilution phase. Subsequently (days 18–26), working memory was tested using a criteria-driven, moving platform paradigm.

taining 4% isoflurane in 100% oxygen. A nose cone with spontaneous ventilation was used for the maintenance of anesthesia utilizing 1.5–2.0% isoflurane in 21% oxygen. Animals were recovered for subsequent testing.

For the cerebral oxygenation studies, rats were initially anesthetized in a plastic box containing 4% isoflurane and 100% oxygen, tracheotomized with a 14-gauge catheter, and ventilation controlled. Anesthesia was maintained with 1.5–2% isoflurane in 21% oxygen and α-chloralose (80 mg/kg) injected into the axillary fat pad before craniotomy. Isoflurane was discontinued after craniotomy, and a period of 45 min was allowed to pass to allow for the elimination of isoflurane and for stabilization of the brain tissue before data collection. α-Chloralose was utilized in this experiment because it has minimal effects on cerebrovascular reactivity as compared with its volatile anesthetic counterparts. α-Chloralose has been used extensively in research for brain functional activation studies because responses appear to mimic the awake state.[26,27] Anesthetic management did not include muscle relaxants.

Hemodilution and Sham Hemodilution Protocols

Under sterile conditions, an incision over the right groin was made. The right femoral vein was cannulated with polyethylene-10 tubing that was fixed in place with suture for the hemodilution process and for blood sampling for hemoglobin and hematocrit. The isovolemic hemodilution process involved three weight-based withdrawals of 2–5 ml (7 ml/kg) venous blood. For the YAn, MAn, and AAn groups, the blood removed was immediately replaced with warmed normal saline at 37°C, in a ratio of 1: 3 (blood:normal saline). For the sham groups (YSh, MSh, and ASh), the withdrawn blood was immediately reinjected. The hemodilution protocol was designed to decrease the hemoglobin concentration in a

step-wise fashion from a baseline of ≈12 g/dl to a final level of ≈5 g/dl after the third hemodilution. Hematocrit and hemoglobin were measured after each hemodilution utilizing a Stat Profile pHOx system (Nova Biomedical, Waltham, MA). The sham hemodilution groups allowed us to control for anesthetic exposure, hypotension related to experimental hemorrhage, surgical stress, and wound-related disability.

For animals in the memory testing and molecular markers of hypoxia experiments, after hemodilution was completed, the groin incision was closed with absorbable suture and the anesthetic discontinued. Animals were recovered under warming lights in cages in the laboratory. When animals demonstrated normal ambulation they were returned to the animal facility.

Visuospatial Learning and Memory Testing

Postoperative cognitive dysfunction typically affects working memory and learning, while sparing long-term or reference memory. Therefore, the impact of aging and isovolemic anemia upon visuospatial reference memory, as well as working memory and learning, was tested, using Morris water maze paradigms designed to differentiate among these specific memory categories. In the Morris water maze, animals are challenged to swim in a circular tank, partially filled with opaque water, using primarily visual cues located in the testing room, to find a submerged platform upon which they can escape. Final group sizes then were: 1) YSh, n = 12; 2) YAn, n = 13; 3) ASh, n = 14; and 4) AAn group, n = 15.

Prehemodilution and posthemodilution visuospatial reference memory was tested through the assessment of performance on a fixed position, submerged platform paradigm, after a period of prehemodilution training on this same platform location. Posthemodilution visuospatial working memory and learning were tested using a criteria-driven, moving

platform paradigm described by Chen *et al.*[28] Success in this latter paradigm demands utilization of attention, conceptual, and procedural working memory to achieve learning upon acutely presented and changing spatial information.

Prehemodilution flagged (cued) training, using a fixed platform location, occurred during days 1–5 and was followed by noncued fixed platform training on days 8–11. Prehemodilution reference memory testing then occurred on day 12 and included measurement of escape latency, swim speed, and distance traveled while searching for a fixed, submerged platform. Reference memory was further tested using a single "probe" trial and was also performed on day 12, where time, percentage of time spent in each quadrant, and platform crossings were recorded while the animal searched for a submerged platform that had been removed from its previously located and trained upon quadrant. Surgery and hemodilution occurred on day 15. Posthemodilution reference memory performance was tested on day 17, using the same performance measures listed above and using the identical platform location used for prehemodilution training and testing. Posthemodilution visuospatial working memory and learning were then tested in a longitudinal fashion on days 18–26, using the criteria-driven, working memory and learning-sensitive moving platform paradigm (see fig. 1).

Definition of Water Maze Measurements
A. Reference Memory.

1. Escape latency(s): time between leaving the starting point and climbing onto the escape platform.
2. Probe testing.
   a. Distance (cm): distance traveled from starting point to platform escape.
   b. Platform crossings (#): number of crossings over the escape platform.
   c. Quadrant time percent (%): the percentage of time spent in the quadrant of the escape platform.

B. Visuospatial Working Memory and Learning. Trials-to-criteria (#): number of trials required to reach working memory success criteria.

Measuring Cerebral Oxygenation and Blood Flow

Anemia has been reported to impair cerebral tissue oxygenation and could contribute to cognitive impairment through this mechanism. We therefore measured the effect of hemodilution upon brain tissue oxygenation and blood flow. The parietal association cortex was studied because of its documented role in visuospatial memory acquisition[29] and ease of access *via* craniotomy. Experimental groups were as follows: YAn, n = 14; AAn, n = 16. A 450-μm-diameter Optronix multiparameter probe (Oxford Optronix, Oxford, United Kingdom) was placed 2–4 mm into the parietal association cerebral cortex, allowing continuous measurement of laser Doppler flow and brain tissue partial pressure of oxygen ($P_{Br}O_2$). Mean arterial pressure (MAP) and rectal temperature were also measured continuously. Arterial blood gas and hemoglobin measurements were made after each hemodilution step and ventilation adjusted to maintain arterial partial pressure of carbon dioxide ($P_{art}CO_2$), 38–42 mmHg.

Molecular Markers of Hypoxia

Quantitative Real-time Polymerase Chain Reaction. N = 10 animals in each of three age groups (Y, M, A) underwent isovolemic (An) or sham (Sh) hemodilution protocols. At 24 or 48 h after hemodilution, the animals were sacrificed and their brains retrieved for RNA extraction. Primers against 18S RNA (Applied Biosystems, Inc., Foster City, CA) were used as endogenous controls for ΔC_T calculations. Primer sets were generated against rat HIF-1α, HIF-2α, carbonic anhydrase-9, erythropoietin, erythropoietin receptor, GLUT-1, PGK-1, VEGF, endothelial nitric-oxide synthase (NOS), inducible NOS, and neuronal NOS.

Western Immunoblotting. N = 3–4 animals in each of three age groups (Y, M, A) underwent isovolemic (An) or sham (Sh) hemodilution protocols. Animals were sacrificed at 24 or 48 h and cortical and hippocampal tissue obtained. Samples from the 3–4 animals in each group were pooled before immunoblot analysis for HIF-1α, VEGF, GLUT-1, and neuronal NOS. Samples for Western blotting were repeated several times to ensure consistency; the graphs presented are representative of those results.

Statistical Analysis

For memory testing, a repeated measures analysis of variance (ANOVA) approach was used to test for intragroup differences over time. Intergroup comparisons utilized the Tukey-Kramer HSD (Tukey-HSD) methodology for multiple comparisons. For the analysis of the role of group (aged *vs.* young) and treatment subgroup (anemia *vs.* sham), a linear mixed-effects model was implemented.

For cerebral oxygenation and blood flow data, intragroup means were compared *via* repeated-measures ANOVA. Comparison of intergroup means was conducted using the paired Student *t* test.

For molecular markers of hypoxia, intragroup means were tested with a two-tailed Student *t* test. ANOVA regression analysis was used to test the dependence of each gene level upon: 1) An *versus* Sh treatment, 2) age group, 3) the interaction between the treatment and the age group (An × Age Grp), 4) the sampling time (24 *vs.* 48 h), and 5) the site of sampling (hippocampus *vs.* cortex).

For all analyses, significance is cited for $P < 0.05$. All analyses were performed with the JMP Statistical Discovery Software (SAS, Inc., Cary, NC). Means ± SD are reported in the results section.

Additional details on all experimental methods can be found in Supplemental Digital Content 1, http://links.lww.com/ALN/A613.
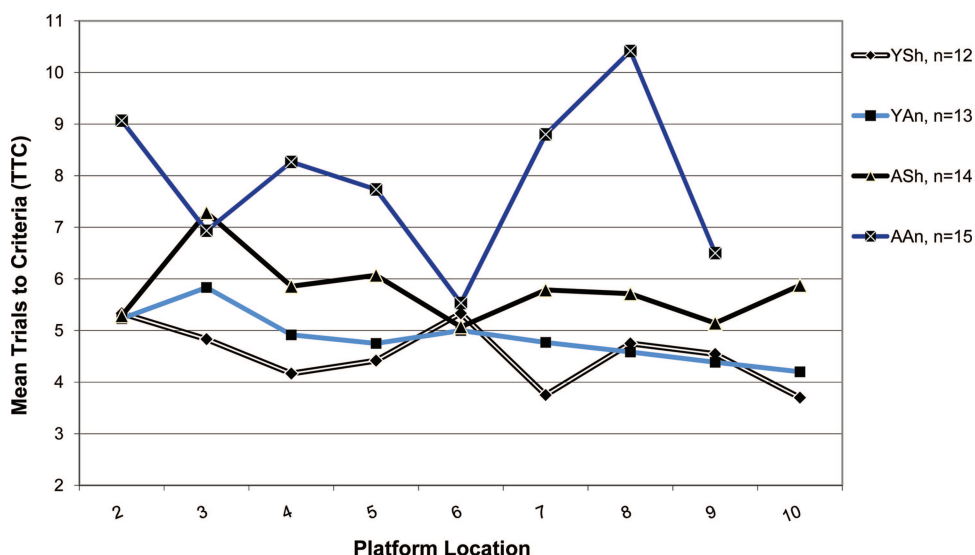
Fig. 2. Working memory testing, using a criteria-driven, moving platform paradigm. The criterion for advancing to a new platform position on the next day was reaching the platform position in less than 15 s, for three consecutive trials. If the criterion was not reached within eight trials, the rat would again be tested again the next day with the same platform location and with eight trials per day, until the criterion was reached. The mean number of trials to criteria (TTC) is plotted by group and by platform location. YAn = aged anemic; ASh = aged sham; Yan = young anemic; YSh = young sham.

Results

Memory Testing
Hemodilution Impaired Visuospatial Working Memory and Learning in the Aged SHR. The sham hemodilution group hemoglobin levels averaged 12.7 ± 0.6 g/dl, and the experimental hemodilution group hemoglobin levels averaged 4.7 ± 0.4 g/dl after treatment. Prehemodilution, posthemodilution, and final recovery hemodilution hemoglobin levels by group can be found in tabular format under Supplemental Digital Content 2,

http://links.lww.com/ALN/A614. Swim speeds can be found in tabular format under Supplemental Digital Content 3, http://links.lww.com/ALN/A615.

The mean number of trials-to-criteria by subgroup and platform location are graphed in figure 2 and summarized in tabular format in Supplemental Digital Content 4, http://links.lww.com/ALN/A616. The figure in Supplemental Digital Content 5 demonstrates percentage failures per day for each subgroup, http://links.lww.com/ALN/A617. The mean
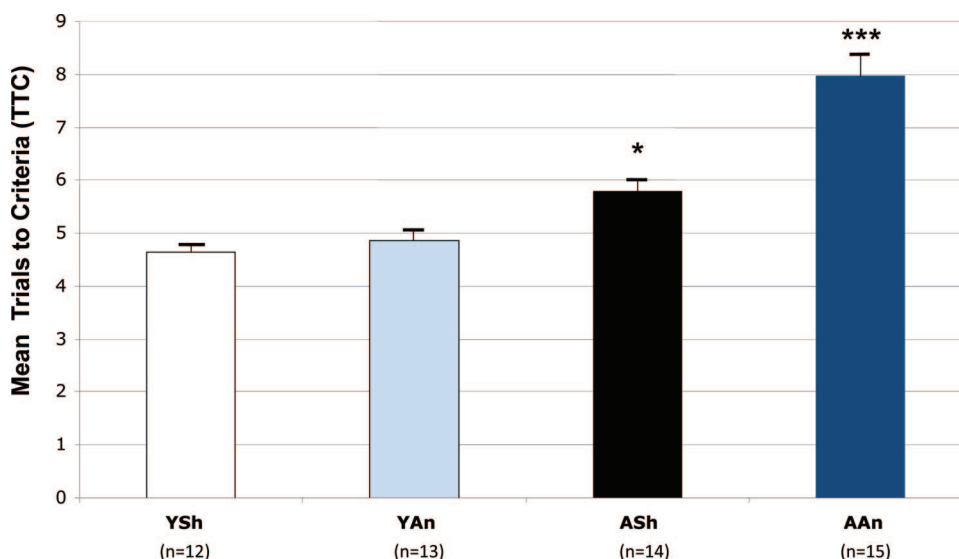

Fig. 3. Working memory testing summary. Mean number of trials to criteria (TTC), averaged over posthemodilution days 18–24. Results are plotted by group. The mean numbers of TTC for platform locations 2–10 were as follows: YSh (4.6 ± 1.7), YAn (4.9 ± 1.7, $P = 0.86$), ASh (5.8 ± 2.3, $P < .05$, *), and AAn (8.0 ± 4.0, $P < 0.001$, ***). Significance is expressed in relationship to YSh performance. AAn = aged anemic; ASh = aged sham; YAn = young anemic; YSh = young sham.

number of trials-to-criteria for platform locations 2–10, figure 3, were as follows: ASh (5.8 ± 2.3), AAn (8.0 ± 4.0), YSh (4.6 ± 1.7), and YAn (4.9 ± 1.7). We tested the dependence of trials-to-criteria upon age group (young *vs.* aged) and treatment subgroup (An *vs.* Sh). Working memory and learning were found to be impaired by aging (age group, $P < 0.001$), by hemodilution (An *vs.* Sh, $P < 0.001$), and by the age group × treatment interaction (AAn *vs.* YAn, $P < 0.01$). Further statistical details describing group differences are summarized in tabular format under Supplemental Digital Content 6 and 7, http://links.lww.com/ALN/A618 and http://links.lww.com/ALN/A619, respectively.

Hemodilution Did Not Impair Reference Memory. Following swim speed can account for the impact of anemia on fatigue and lethargy, independent of potential cognitive effects. Average posthemodilution swim speed (cm/s) slowed markedly for ASh and AAn, from an average of 36.4 ± 7.4 cm/s to 29.0 ± 7.6 cm/s ($P < 0.001$), now slower for the aged animals than for the younger groups, 32.9 cm/s ($P < 0.001$), the opposite of what had been seen in the prehemodilution phase (Supplemental Digital Content 3, http://links.lww.com/ALN/A615). Although anemia may have resulted in greater fatigue in older animals, there remained no important difference in swim speed between the ASh and AAn subgroups ($P = 0.75$), while there was a small difference between the YSh and YAn subgroups ($P < 0.001$) after the sham or anemic treatments. Escape latency, reported as the average of four trials to the hidden platform for P-1 on the first day of testing posthemodilution (Day 16), is listed in Supplemental Digital Content 8, where reference memory variables are summarized in tabular format for prehemodilution and posthemodilution phases, http://links.lww.com/ALN/A620. All groups demonstrated roughly parallel increases in latency from prehemodilution to posthemodilution phases. Within-group analysis (YSh *vs.* YAn, or ASh *vs.* AAn) of latency measures demonstrated no significant differences (Supplemental Digital Content 8, http://links.lww.com/ALN/A620). Distance traveled increased in all subgroups, and remained increased in the aged animals relative to younger cohorts. Posthemodilution probe testing revealed no remarkable decrement in platform crossings, quadrant time, or quadrant time percent from the prehemodilution phase.

Cerebral Oxygenation
Anemia Does Not Impair Local Cerebral Cortical Tissue Oxygenation in Young or Aged SHR. Chronic hypertension and atheromatous changes in the vasculature accelerate with aging, which may render tissue especially vulnerable to anemic hypoxia. We therefore tested the impact of isovolemic anemia upon local brain partial pressure of oxygen and laser Doppler flow in young (YAn) and aged (AAn) male SHR.

A summary of the results of bivariate analyses for all physiologic measurements is shown in table 1. Surpris-

Table 1. Comparisons of Physiologic Parameters at Four Stages of Graded Hemodilution

	YAn (n = 14)	AAn (n = 16)	P
Baseline			
Hgb, g/dl	11.5 ± 1.0	12.2 ± 1.0	0.05*
Ca_{O_2}, ml/dl	15.6 ± 1.5	16.4 ± 1.4	0.12
$P_{Art}O_2$, mmHg	84.9 ± 8.5	80.2 ± 8.8	0.15
$P_{Art}CO_2$, mmHg	37.0 ± 4.4	39.3 ± 3.4	0.13
MAP, mmHg	149 ± 19	144 ± 28	0.58
$Temp_R$, °C	37.0 ± 0.4	37.0 ± 0.4	0.96
$Temp_{Br}$, °C	34.1 ± 0.6	34.1 ± 1.1	0.93
LDF, units	889 ± 529	886 ± 534	0.99
$P_{Br}O_2$, mmHg	19.6 ± 6.9	20.0 ± 8.5	0.78
Hemodil #1			
Hgb, g/dl	8.6 ± 0.9	9.2 ± 1.0	0.11
Ca_{O_2}, ml/dl	11.7 ± 1.3	12.4 ± 1.4	0.17
$P_{Art}O_2$, mmHg	86.1 ± 7.9	83.7 ± 10.7	0.50
$P_{Art}CO_2$, mmHg	34.5 ± 2.4	36.1 ± 2.9	0.11
MAP, mmHg	137 ± 17	136 ± 23	0.92
$Temp_R$, °C	37.3 ± 0.3	37.0 ± 0.3	0.05*
$Temp_{Br}$, °C	34.6 ± 0.9	34.1 ± 1.1	0.20
LDF, units	1,199 ± 676	937 ± 480	0.24
$P_{Br}O_2$, mmHg	19.9 ± 9.5	21.3 ± 8.7	0.68
Hemodil #2			
Hgb, g/dl	6.8 ± 0.7	7.3 ± 1.0	0.16
Ca_{O_2}, ml/dl	9.3 ± 0.8	9.9 ± 1.4	0.15
$P_{Art}O_2$, mmHg	88.9 ± 10.7	86.1 ± 10.6	0.48
$P_{Art}CO_2$, mmHg	33.7 ± 3.5	35.4 ± 3.4	0.19
MAP, mmHg	123 ± 10	130 ± 23	0.31
$Temp_R$, °C	37.1 ± 0.3	37.3 ± 0.5	0.14
$Temp_{Br}$, °C	34.6 ± 0.9	34.8 ± 0.9	0.56
LDF, units	1,176 ± 772	990 ± 454	0.42
$P_{Br}O_2$, mmHg	19.6 ± 10.5	21.7 ± 9.6	0.58
Hemodil #3			
Hgb, g/dl	5.1 ± 0.4	5.5 ± 0.8	0.14
Ca_{O_2}, ml/dl	6.9 ± 0.6	7.6 ± 1.1	0.08
$P_{Art}O_2$, mmHg	88.1 ± 14.0	99.3 ± 15.9	0.05*
$P_{Art}CO_2$, mmHg	33.8 ± 4.0	33.1 ± 2.4	0.56
MAP, mmHg	86 ± 14	116 ± 30	0.003*
$Temp_R$, °C	37.1 ± 0.3	37.0 ± 0.5	0.78
$Temp_{Br}$, °C	34.9 ± 0.9	34.9 ± 1.2	0.87
LDF, units	1,240 ± 803	1,089 ± 471	0.53
$P_{Br}O_2$, mmHg	19.3 ± 10.2	21.4 ± 10.0	0.53

Values are mean ± SD.

* $P < 0.05$.

Ca_{O_2} = arterial oxygen content; Hgb = hemoglobin concentration; LDF = laser Doppler flow; MAP = mean arterial pressure; $P_{Art}CO_2$ = arterial partial pressure of carbon dioxide; $P_{Art}O_2$ = arterial partial pressure of oxygen; $P_{Br}O_2$ = brain partial pressure of oxygen; $Temp_{Br}$ = brain temperature; $Temp_R$ = rectal temperature.

ingly, steady-state tissue brain partial pressures of oxygen values did not change appreciably from baseline values ($P = 0.49$).

We did not attempt to control MAP during this experiment, letting it "float" under the conditions of the experiment. MAP was only significantly different between groups, however, after the third hemodilution, falling by 17 ± 26% in the AAn group and 41 ± 13% in the YAn animals ($P = 0.005$). A positive correlation was apparent between the
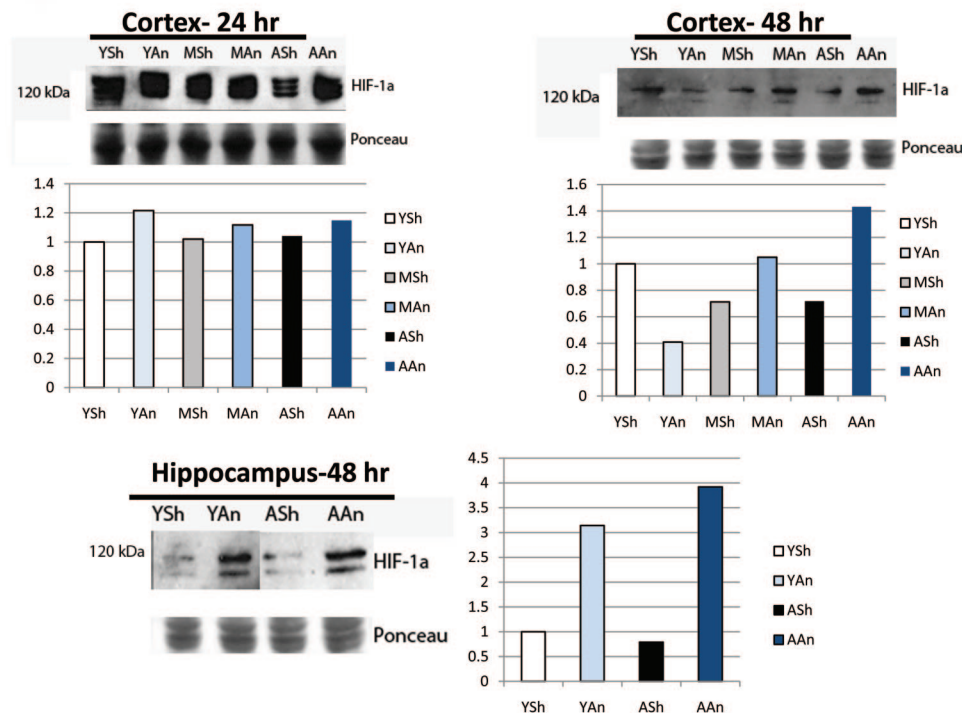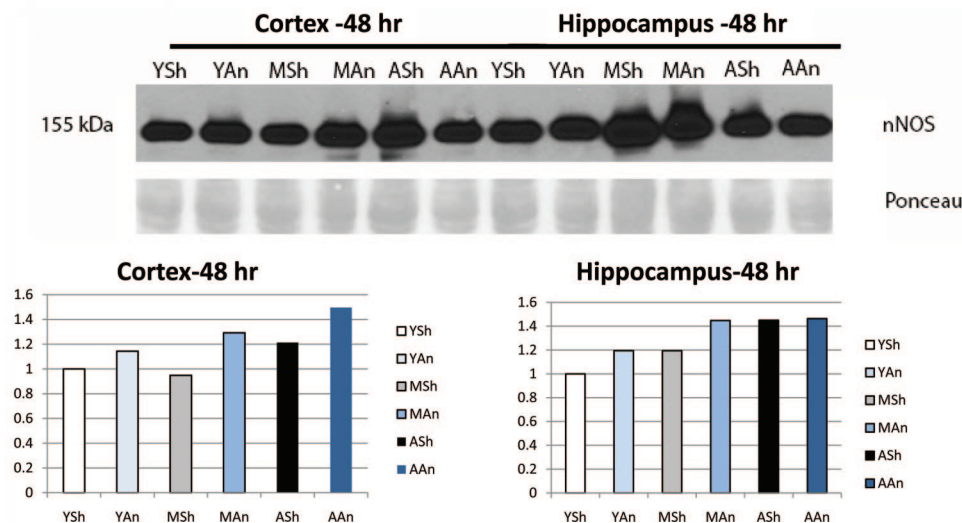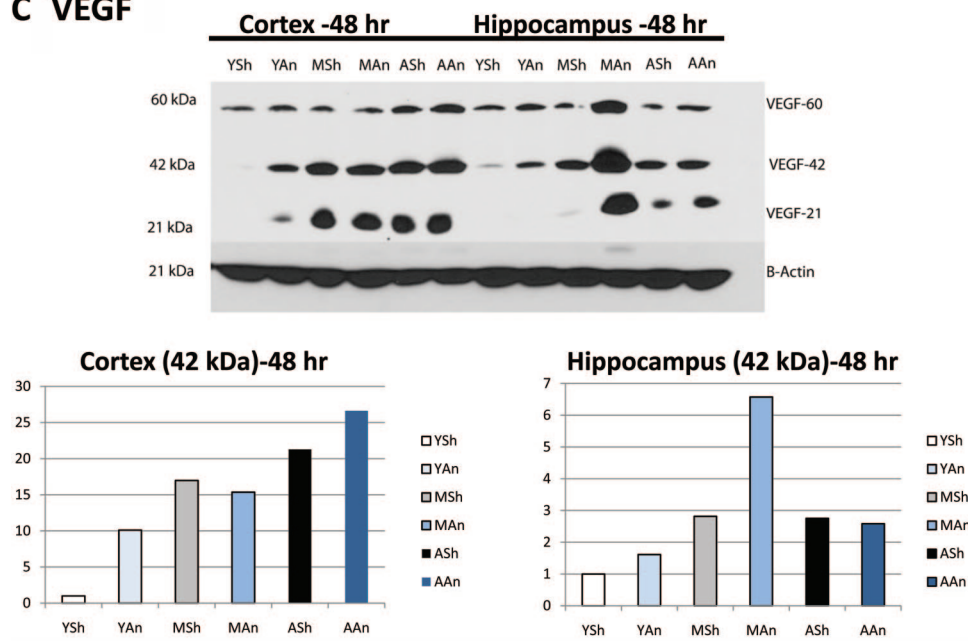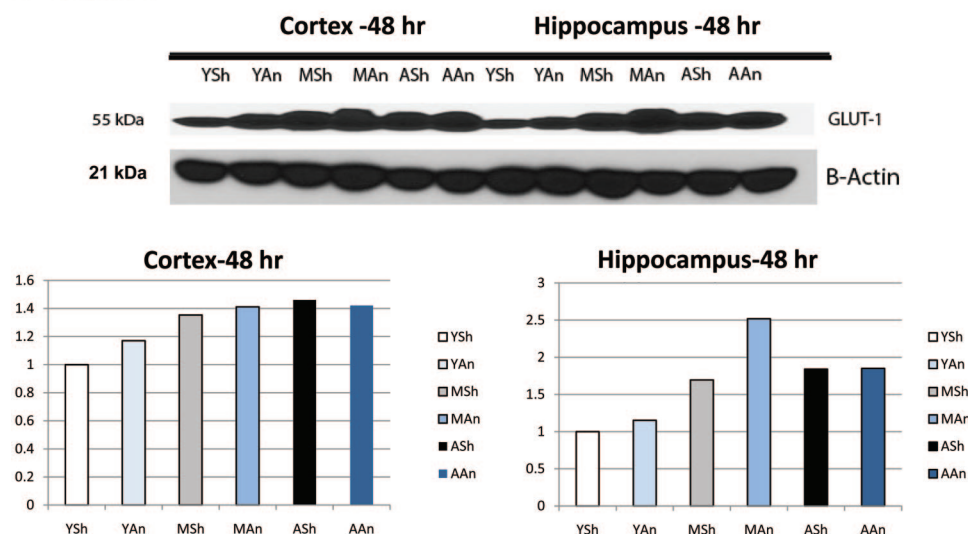
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Fig. 4. Western Immunoblots for (*A*) HIF-1α, (*B*) nNOS, (*C*) VEGF, and (*D*) GLUT-1. N = 3–4 animals per group contributed both cortical and hippocampal samples. Within each group, protein lysates were pooled prior to analysis. Band densities are expressed relative to YSh and adjusted for protein loading with β-actin or Ponceau lane band density. Groups: AAn = aged anemic (n = 4 at 24 h, n = 3 at 48 h); ASh = aged sham (n = 3 at 24 h, n = 3 at 48 h); MAn = mature anemic (n = 4 at 24 h, n = 3 at 48 h); MSh = mature sham (n = 4 at 24 h, n = 3 at 48 h); YAn = young anemic (n = 4 at 24 h, n = 3 at 48 h); YSh = young sham (n = 4 at 24 h, n = 3 at 48 h). Proteins: GLUT-1 = glucose transporter-1; HIF-1α = hypoxia-inducible factor-1α; nNOS = neuronal nitric-oxide synthase; VEGF = vascular endothelial growth factor.

change in brain partial pressure of oxygen that occurred from baseline to completion of the hemodilution protocol and percentage change in MAP for the AAn animals ($P = 0.017$), although none appeared to exist for the younger animals ($P = 0.57$); see figures in Supplemental Digital Content 9, http://links.lww.com/ALN/A621. The slope of the regres-

sion line climbed more steeply for the AAn animals by $\approx$37% relative to YAn animals. This difference persisted (28%) even after the young outlier (red circle) was withdrawn from the analysis.

Laser Doppler flow measurements increased from baseline by 31 $\pm$ 41% for the AAn animals and increased to a

Fig. 4. Continued.

Molecular Markers of Cellular Hypoxia

To test the cellular sensing of and responses to anemic hypoxia, we measured levels of the HIF-1α protein and examined the gene expression profiles for downstream HIF-regulated genes, including HIF-1α, HIF-2α, carbonic anhydrase-9, erythropoietin, erythropoietin receptor, GLUT-1, PGK-1, VEGF, endothelial NOS, and inducible NOS after sham or anemic exposures. The hypoxia-sensitive neuronal NOS gene was also measured. Finally, we also measured translation of HIF-1α, VEGF,

somewhat lesser degree, 17 $\pm$ 28%, for YAn animals, from baseline to final hemodilution, ($P = 0.028$). Stepwise details can be found in table 1.

GLUT-1, and neuronal NOS. Hemoglobin levels for animal groups used in messenger ribonucleic acid (mRNA) and protein analyses are summarized in tabular format in Supplemental Digital Content 10, http://links.lww.com/ALN/A622.

HIF-1α protein levels in the cortex and hippocampus were increased in response to anemia in all age groups, but also appeared to be increased in relatively greater magnitude in aged anemic animals relative to younger cohorts, especially at 48 h in both the hippocampus and cortex; see figure 4a. HIF-1α levels were increased roughly 5-fold in the hippocampus and doubled in the cortex at 48 h. This compared with a 3-fold elevation in the hippocampus, and a decreased level in the cortex, in younger cohorts.
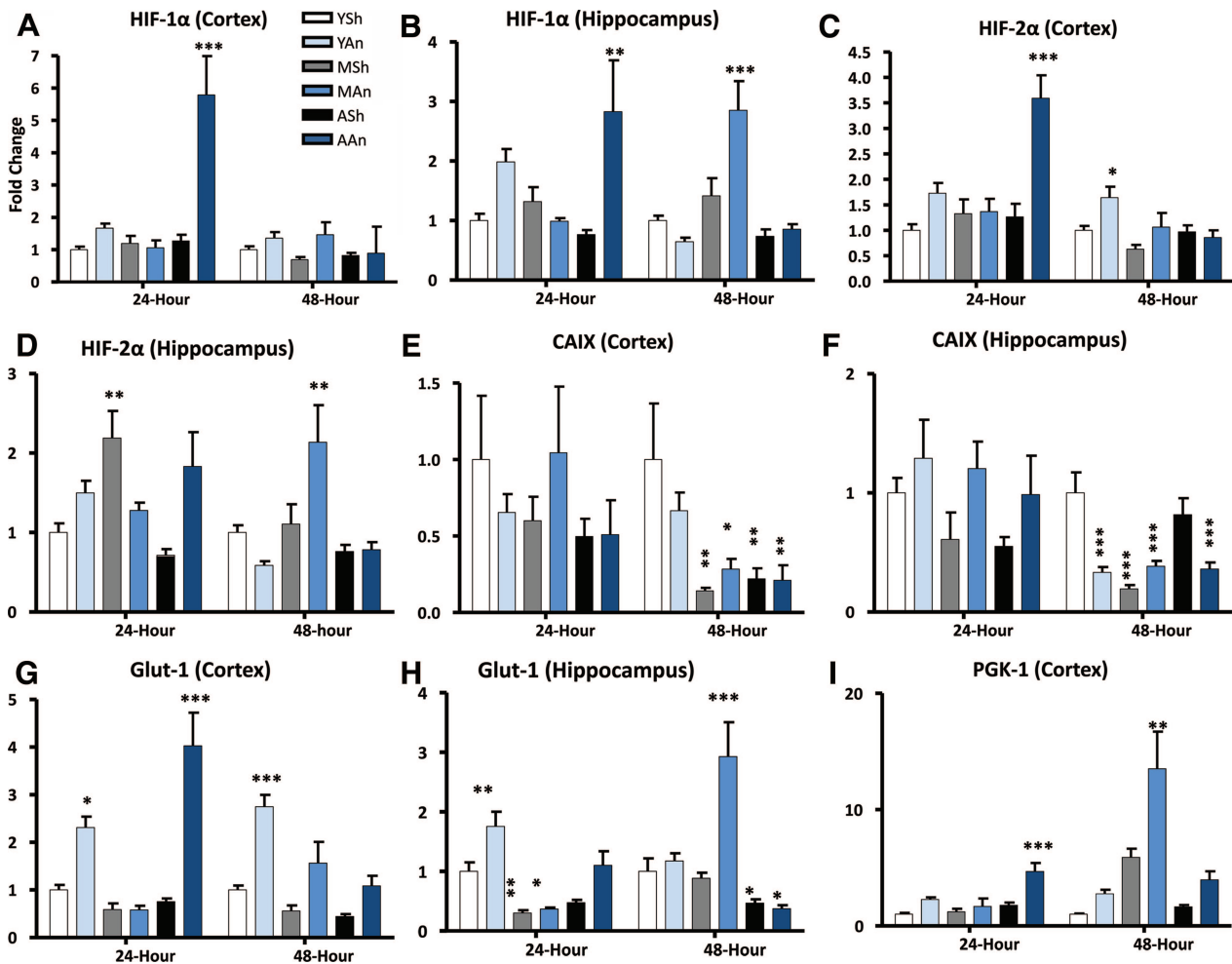
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Fig. 5. Hypoxia sensitive messenger ribonucleic acid (mRNA) levels for cortex and hippocampus. In *A–V,* hypoxia sensitive mRNA levels are presented for 24- and 48-h anemic and sham exposures; n = 10/group. mRNA levels are presented relative to the normalized young sham control group (YSh) at both time points. Significance was tested between all groups and the YSh group using the Student *t* test with Dunnet correction for multiple comparisons. * $P \leq 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Groups: AAn = aged anemic; ASh = aged sham; MAn = mature anemic; MSh = mature sham; YAn = young anemic. Genes: CAIX = carbonic anhydrase-9; eNOS = endothelial nitric-oxide synthase; EPO = erythropoietin; EPOr = erythropoietin receptor; GLUT-1 = glucose transporter-1; HIF-1α = hypoxia-inducible factor-1α; HIF-2α = hypoxia-inducible factor-2α; iNOS = inducible nitric-oxide synthase; nNOS = neuronal nitric-oxide synthase; PGK-1 = phosphoglygerate kinase-1; VEGF = vascular endothelial growth factor.

A subset of HIF-regulated (VEGF and GLUT-1) or hypoxia-regulated proteins (neuronal NOS) were also examined; these results can be found in figure 4, b–d. VEGF and GLUT-1 protein expressions appeared to be increased, and neuronal NOS was depressed in the ASh groups relative to YSh and MSh. Anemia resulted in the increased expression of all tested proteins (neuronal NOS, VEGF, and GLUT-1). In the AAn group, the translation of GLUT-1, a key protein in switching from aerobic metabolism to glycolysis during oxygen deprivation, appeared to be blunted relative to younger cohorts.

HIF- and hypoxia-regulated downstream mRNA levels were measured in the sham and anemic subgroups within each age group. In figure 5, mRNA levels for all groups are compared with normalized YSh mRNA levels. Multiple downstream genes known to be regulated by the HIF-1α and HIF-2α transcription factors, or by hypoxic exposure, were

consistently increased within both the cortex and hippocampus in response to anemia, indicating the sensing of hypoxia in all age groups in response to the anemic challenge.

Table 2 summarizes the ANOVA regression analysis testing the dependence of each gene level upon: 1) An *versus* Sh treatment, 2) age group, 3) the interaction between the treatment and the age group (An *vs.* Sh × Age Grp), 4) the sampling time (24 *vs.* 48 h), and 5) the site of sampling (hippocampus *vs.* cortex).

In table 2, the column labeled An *versus* Sh shows that the coefficients for HIF-1α and HIF-2α and all other genes, except for inducible NOS and carbonic anhydrase-9, were positive and significant, indicating that the anemic exposure resulted in the cellular sensing of hypoxia in all age groups.

In the table 2 column labeled Age Group, significance of the coefficients tests the effect of age group upon the tested
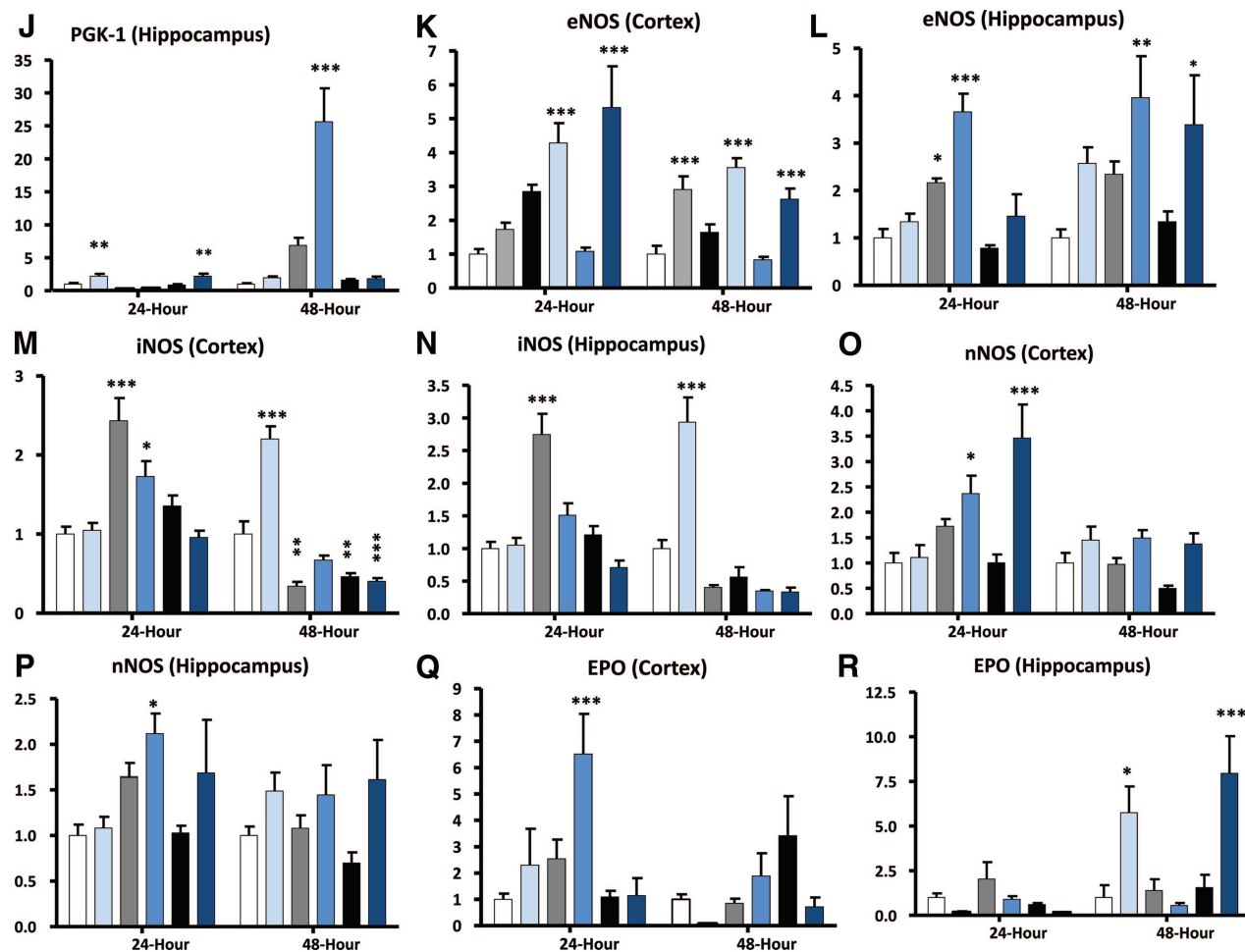
Fig. 5. Continued.

genes in the sham groups. Advanced age in the SHR, independent of any effect of anemic exposure, was associated with increased levels of HIF-1α and HIF-2α mRNA, or an increased sensing of hypoxia, as evidenced by the 31% increase in the HIF-1α coefficient for the ASh group relative to YSh. Erythropoietin receptor and PGK-1 mRNA levels were markedly increased in the mature group (M) relative to the young (Y) animals.

Aging impacted the transcriptional response to the anemic exposure. In table 2, in the column labeled An × Age Grp, we tested the interaction (×) of the effect of anemic exposure within the three age groups. The AAn group experienced a greater elevation of HIF-1α and HIF-2α genes relative to both MAn and YAn groups, particularly at 24 h, offering further support for the conclusion that anemia results in more cellular hypoxia with aging in the SHR. Anemic exposures in the aged (AAn) animals also resulted in elevation of several HIF-regulated or hypoxia-sensitive downstream gene products, including VEGF, endothelial NOS, and neuronal NOS. In contrast, in response to the anemic exposures, levels of HIF-1α and HIF-2α mRNA were not significantly different between the YAn and MAn groups.

Discussion

In response to exposure to acute isovolemic anemia, we have demonstrated the following: 1) evidence of age-dependent working memory and learning dysfunction which spared reference memory, 2) an unimpaired local cerebral tissue blood flow response and maintenance of tissue oxygenation with aging, 3) molecular evidence of cellular sensing of hypoxia in spite of normal systemic (arterial) and tissue oxygen levels, and 4) an increased sensing of cellular hypoxia with aging.

Working memory, defined as the ability to temporarily store, manipulate, and process new information,[30] appears requisite for hippocampal based learning.[31,32] Working memory involves multiple brain regions, including the prefrontal lobe, parahippocamal structures, parietal association cortex, and subcortical network (thalamic nuclei, limbic system).[33,34] Although the hippocampus and mesial temporal lobe are considered critical for learning and memory, they also appear to have an important role in working memory.[35] Postoperative cognitive dysfunction primarily affects the working memory domains of attention, cognitive speed, and executive function, as well as hippocampal based memory acquisition or learning.[1] The impairment of visuospatial working memory and learning but not
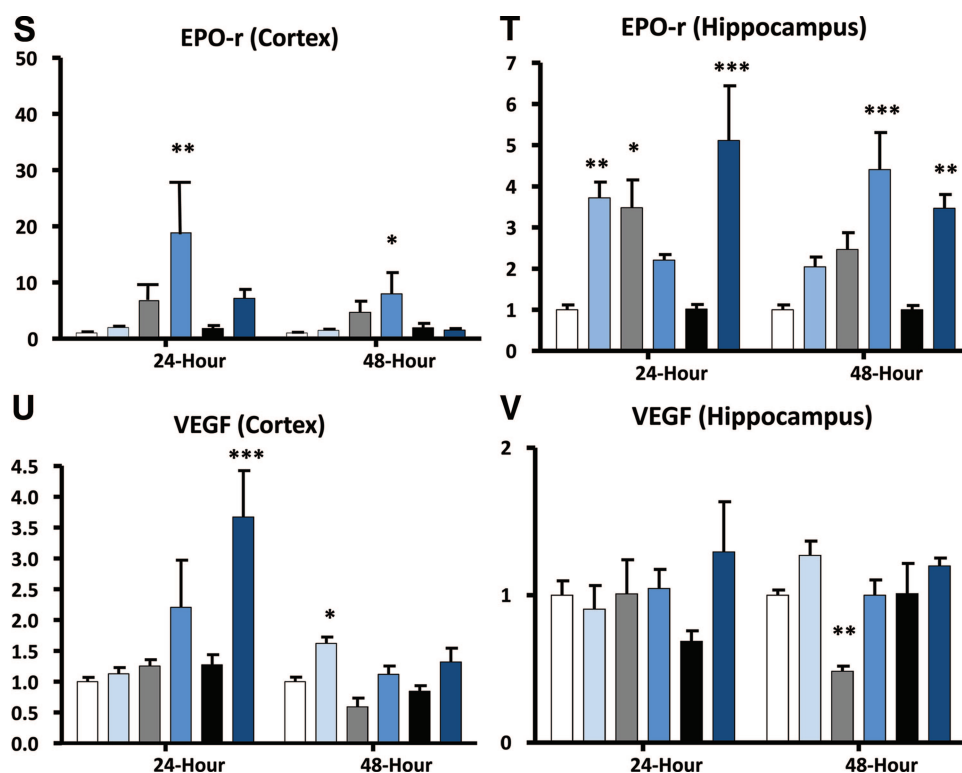
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Fig. 5. Continued.

visuospatial reference memory in our translational model is consistent with the human experience.[1,36]

Acute isovolemic anemia does not impair cerebral tissue oxygenation, as measured with an invasive cortical tissue oxygen probe, even with aging and chronic hypertension. The absence of an aging impact upon local cerebral oxygenation with hemodilution was unexpected, especially when using the SHR model. Although hemodilution resulted in stable cerebral oxygenation in both young and aged SHR groups, it should be noted that this was so in the younger group in spite of a 41% decrease in MAP. Stable $P_{Br}O_2$ levels in the aged SHR group were accompanied by a much smaller 17% decrease in MAP. The figures provided in Supplemental Digital Content 9, http://links.lww.com/ALN/A621, with graphs of $P_{Br}O_2$ *versus* MAP for both YAn and AAn animals, demonstrate that there may indeed exist a steeper MAP:$P_{Br}O_2$ relationship with anemia in the setting of chronic hypertension.

The demonstration of a seemingly adequate increase in brain blood flow and stable tissue oxygen levels equates only with adequate local oxygen delivery, however. Invasive tissue oxygen probes, such as we employed and such as are used clinically, measure a milieu with contributions from the arteriole, the capillary, the cell, and the venule and may therefore poorly reflect the status of the cell. Further, we were able to sample only one site in the brain. The age- and hypertension-dependent up-regulation of molecular markers of cellular hypoxia, when oxygen delivery is apparently stable and tissue perfusion luxuriant relative to younger cohorts, may suggest that brain oxygen extraction, under conditions of acute isovolemic anemia, may be flow-dependent in the

chronically hypertensive brain. An impairment in microvascular compliance and distribution of blood flow during isovolemic anemia with chronic hypertension could also be hypothesized. Finally, these measurements were conducted under the anesthetic influence of α-chloralose, after the discontinuation of isoflurane. α-Chloralose has been used extensively in animal research, including in experiments on functional magnetic resonance imaging and flow-activation coupling, because this anesthetic appears to cause minimal perturbations in cerebral oxygen metabolism and cerebrovascular reactivity. We recognize that this approach may not, and cannot exactly, reflect the truly awake state.

The prolyhydroxylase family of proteins interacts closely with mitochondria to sense cellular hypoxia at the level of the cell.[19] Hare[37] and McLaren[38] first documented, and we now confirm, that clinically relevant levels of acute isovolemic anemia initiate cerebral cellular molecular changes in hypoxia-related gene synthesis and protein translation, in the absence of systemic (arterial) or tissue hypoxia as measured with an invasive cortical tissue oxygen probe. The up-regulation of HIF and related molecular biomarkers of hypoxia may be triggered at oxygen tensions higher than previously thought to be associated with cellular hypoxia. Lopez-Lazaro[39] has coined the term dysoxia-inducible factor as an alternative to HIF. HIF may be up-regulated or stabilized by subtle changes in basal cellular oxygen metabolism, which in turn may be controlled by subtle changes in the perceived availability of oxygen supply.[40] Thus, the cell appears to initiate life-saving strategies in response to even mild, suble-

Table 2. Gene Level Dependence upon Sham (Sh) *vs.* Anemic (An) Exposure, Age Group, Anemia × Age Group Interaction, Duration of Anemic Exposure, and Brain Region Tested

Gene	Sham *vs.* Anemic Exposures	Age Group		Anemia × Age Group		24- *vs.* 48-h Exposures	Hippocampus *vs.* Cortex
HIF-1α	0.43 (0.09)***	0.31 (0.13)*	A	0.42 (0.13)**		−0.60 (0.18)**	0.08 (0.09)
		−0.07 (0.13)	M	−0.20 (0.13)			
HIF-2α	0.23 (0.06)***	0.043 (0.08)	A	0.19 (0.08)*		−0.52 (0.11)***	0.07 (0.06)
		0.087 (0.08)	M	−0.16 (0.08)			
VEGF	0.28 (0.06)***	0.21 (0.08)*	A	0.19 (0.08)*		−0.34 (0.11)**	0.21 (0.06)***
		−0.12 (0.08)	M	−0.027 (0.08)			
EPO	0.44 (0.21)*	0.19 (0.30)	A	−0.0046 (0.30)		0.56 (0.43)	−0.03 (0.21)
		0.19 (0.30)	M	−0.084 (0.30)			
EPO-r	1.29 (0.42)**	−0.70 (0.59)	A	0.14 (0.59)		−1.7 (0.83)*	1.02 (0.42)*
		2.7 (0.59)***	M	0.49 (0.59)			
e-NOS	0.83 (0.10)***	−0.13 (0.14)	A	0.29 (0.14)*		0.032 (0.20)	0.16 (0.10)
		0.81 (0.14)***	M	−0.027 (0.14)			
i-NOS	0.029 (0.05)	−0.42 (0.07)***	A	−0.15 (0.07)*		−0.50 (0.10)***	−0.009 (0.05)
		0.17 (0.07)*	M	−0.23 (0.07)**			
n-NOS	0.34 (0.06)***	0.037 (0.08)	A	0.28 (0.08)***		−0.43 (0.12)***	0.065 (0.06)
		0.22 (0.08)	M	−0.09 (0.08)			
CA-9	0.03 (0.05)	−0.13 (0.07)	A	−0.016 (0.07)		−0.23 (0.10)*	−0.36 (0.05)***
		−0.09 (0.07)	M	0.16 (0.07)*			
GLUT-1	0.49 (0.07)***	−0.08 (0.10)	A	0.084 (0.10)		−0.0008 (0.14)	0.20 (0.07)**
		−0.22 (0.10)*	M	−0.095 (0.10)			
PGK	1.6 (0.37)***	−1.3 (0.52)*	A	−0.77 (0.52)		4.0 (0.73)***	−0.18 (0.37)
		3.3 (0.52)***	M	1.8 (0.52)***			

Statistics: Employing ANOVA multiple regression analysis, coefficients derived include the following.

Sham *vs.* Anemic exposures: Tests the significance of the effect of anemia relative to the sham exposures in controlling the specific gene level.

Age Group: Tests for significant differences between the aged (A) *vs.* young, and mature (M) *vs.* young age groups in controlling the specific gene level, independent of, or after controlling for, the effect of anemia. Coefficients and significance levels are expressed relative to the young group.

Anemia × Age group: Tests for the effect of anemic *vs.* sham exposures within each age group. Coefficients and significance levels are relative to the young anemic group.

24-h *vs.* 48-h Exposures: Tests for differences in gene levels by duration of anemic or sham exposures. Coefficients and significance levels are relative to 24-h exposure.

Hippocampus *vs.* Cortex: Tests for differences in gene levels by tissue sampled. Coefficients and significance levels are relative to cortex.

Standard error is expressed in parentheses.

Significance is denoted in the following manner (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

CA-9 = carbonic anhydrase-9; EPO = erythropoietin; EPO-r = erythropoietin receptor; GLUT-1 = glucose transporter-1; HIF-1α = hypoxia-inducible factor-1α; HIF-2α = hypoxia-inducible factor-2α; NOS = nitric-oxide synthases (e = endothelial, i = inducible, and n = neuronal); PGK-1 = phosphoglycerate kinase-1; VGEF = vascular endothelial growth factor.

thal hypoxia, and we must consider the potential impact of this strategy upon cognitive processes.

Aging with chronic hypertension was associated with a markedly increased cellular sensing of hypoxia relative to younger cohorts, as evidenced by the pronounced upregulation of HIF-1α protein, and HIF-1α and HIF-2α genes. Genes and proteins downstream of HIF-1α and HIF-2α, or responsive to hypoxic stimuli (neuronal NOS), however, demonstrated a mixed response. VEGF, endothelial NOS, and neuronal NOS genes were markedly increased in the AAn group relative to MAn and YAn, but most others, including erythropoietin, erythropoietin receptor, GLUT-1, and PGK-1, did not follow suit. Overall, this may indicate a post-transcriptional or translational failure to synthesize key proteins with aging in response to hypoxia. Previous work in nonhyper-tensive animals has demonstrated an impaired HIF-regulated and hypoxia-sensitive gene and protein expression upon hypoxic exposure with aging.[23,41–44] We have studied a model where the impact of hypertension upon oxygen delivery and extraction is superimposed upon this aging background. Under these circumstances, therefore, one could hypothesize that anemia might cause a greater degree of hypoxia and a diminished ability to manage the threat successfully.

Consistent with reports of only temporary cognitive impairment after surgery, with fairly consistent reports of recovery at 6 weeks to 3 months, hypoxia need not result in cell death to impact cognition. Working memory and learning (memory consolidation) may be impaired by a temporary bottleneck in protein synthesis.[45] The dependence of working memory[46] and neuronal plasticity[47] upon new protein

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synthesis, to include probably the most important protein in this process, brain-derived neurotrophic factor, is well documented. The down-regulation of brain-derived neurotrophic factor during exposure to hypoxia,[48] chronic anemia,[49] and chronic hypertension[50] has also been documented, as has been the dependence of brain-derived neurotrophic factor recovery upon erythropoietin synthesis,[51] and an age-related impairment in erythropoietin response to hypoxia.[52] Finally, the tight dependence of erythropoietin synthesis upon HIF-2α in the brain[53] more clearly defines just why mild hypoxia, or anemic hypoxia, might impact working memory and learning in a temporary fashion and why an age-dependent impairment in the ability to synthesize erythropoietin and brain-derived neurotrophic factor might further impact working memory and learning.

In summary, in a translational animal model of aging upon a background of chronic hypertension, acute anemia resulted in age-dependent working memory and learning impairment, similar to the type of cognitive deficit that is seen clinically in humans with similar comorbidities. This was matched by an age-dependent increase in molecular markers of cellular hypoxia. Finally, molecular changes consistent with the sensing of hypoxia at the level of the cell may occur in the absence of systemic and cerebral hypoxia when measured *via* invasive tissue oxygen probes. A potential role for anemia in POCD and the possible mechanism of cellular hypoxia is highly relevant in cardiac and noncardiac surgery arenas, especially in the aged, chronically hypertensive patient. The discordance of the tissue oxygen probe data with cellular biomarkers of hypoxia and with the cognitive impairment are particularly relevant in light of the growing utilization of comparable brain tissue oxygen monitors in neurointensive care and the use of surface cerebral oximetry probes in the operating room for the prevention of brain injury.

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