Differential HIF and NOS responses to acute anemia: defining organ-specific hemoglobin thresholds for tissue hypoxia

Albert K. Y. Tsui,1,2 Philip A. Marsden,2,4 C. David Mazer,1,2,3 John G. Sled,5 Keith M. Lee,1 R. Mark Henkelman,5 Lindsay S. Cahill,5 Yu-Qing Zhou,5 Neville Chan,1 Elaine Liu,1,2 and Gregory M. T. Hare*1,2,3

1Department of Anesthesia, St. Michael’s Hospital, University of Toronto, Ontario, Canada; 2Keenan Research Centre for Biomedical Science and Li Ka Shing Knowledge Institute of St. Michael’s Hospital, Toronto, Ontario, Canada; 3Department of Physiology, University of Toronto, Toronto, Ontario, Canada; 4Department of Medicine, Division of Nephrology, St. Michael’s Hospital, University of Toronto, Toronto, Ontario, Canada; 5Department of Medical Biophysics, University of Toronto, Mouse Imaging Centre, Hospital for Sick Children, Toronto, Ontario, Canada

Submitted 28 August 2013; accepted in final form 18 April 2014

Tsui AK, Marsden PA, Mazer CD, Sled JG, Lee KM, Henkelman RM, Cahill LS, Zhou Y, Chan N, Liu E, Hare GM. Differential HIF and NOS responses to acute anemia: defining organ-specific hemoglobin thresholds for tissue hypoxia. Am J Physiol Regul Integr Comp Physiol 307: R13–R25, 2014. First published April 23, 2014; doi:10.1152/ajpregu.00411.2013.—Tissue hypoxia likely contributes to anemia-induced organ injury and mortality. Severe anemia activates hypoxia-inducible factor (HIF) signaling by hypoxic- and neuronal nitric oxide (NO) synthase-(nNOS) dependent mechanisms. However, organ-specific hemoglobin (Hb) thresholds for increased HIF expression have not been defined. To assess organ-specific Hb thresholds for tissue hypoxia, HIF-α (oxygen-dependent degradation domain, ODD) luciferase mice were hemodiluted to mild, moderate, or severe anemia corresponding to Hb levels of 90, 70, and 50 g/l, respectively. HIF luciferase reporter activity, HIF protein, and HIF-dependent RNA levels were assessed. In the brain, HIF-1α was paradoxically decreased at mild anemia, returned to baseline at moderate anemia, and then increased at severe anemia. Brain HIF-2α remained unchanged at all Hb levels. Both kidney HIF-1α and HIF-2α increased earlier (Hb 70–90 g/l) in response to anemia. Liver also exhibited an early HIF-α response. Carotid blood flow was increased early (Hb 70 g/l), but renal blood flow remained relatively constant, only increased at Hb of 50 g/l. Anemia increased nNOS (brain and kidney) and endothelia NOS (eNOS) (kidney) levels. Whereas anemia-induced increases in brain HIFα were nNOS-dependent, our current data demonstrate that increased renal HIFα was nNOS independent. HIF-dependent RNA levels increased linearly (~10-fold) in the brain. However, renal HIF-RNA responses (MCT4, EPO) increased exponentially (~100-fold). Plasma EPO levels increased near Hb threshold of 90 g/l, suggesting that the EPO response is sensitive. Collectively, these observations suggest that each organ expresses a different threshold for cellular HIF/NOS hypoxia responses. This knowledge may help define the mechanism(s) by which the brain and kidney maintain oxygen homeostasis during anemia.

anemia; hemoglobin threshold; hypoxia inducible factor; nitric oxide synthase

ANEMIA IS A GLOBAL HEALTH PROBLEM that affects nearly a quarter of the world’s population. Although anemia is a multietiology disease, it is commonly associated with increased organ injury and mortality in many populations including trauma and surgical patients who experience acute blood loss (3, 7, 35) and those with chronic diseases (1, 47). Specifically, preoperative and interoperative anemia are accurate predictors of adverse outcomes in surgical patients, including cerebral injury, stroke, and renal failure (19, 33–36). Despite the fact that the current treatments of anemia with transfusion and erythropoiesis (EPO) stimulating agents lead to an increase in hemoglobin (Hb) concentration, they do not provide survival benefits (11, 23, 47, 52). The problem is compounded by the fact that the optimal Hb trigger for the treatment of anemia is debated (8, 20). This clinical paradox highlights the need for a clearer understanding of the adaptive responses to anemia, and the Hb thresholds at which they are activated.

At the physiological level, anemia leads to activation of compensatory cardiovascular mechanisms to optimize tissue oxygen delivery. Redistribution of blood flow occurs such that organs with high metabolic demand (e.g., brain, heart) receive preferential blood flow relative to those with low metabolic demand (e.g., kidney, liver, intestine) (39, 48, 57). Despite activation of these physiological responses, tissue oxygen tension in vital organs (brain) decreases in proportion to the Hb level (56). Interestingly, the threshold for decreased tissue PO2 is not uniform throughout the body. For example, kidney PO2 decreases earlier and at higher Hb concentration compared with the brain (48) and the heart (57) during acute anemia. Tissue oxygen consumption is also heterogeneous and organ specific. Although overall total body oxygen consumption is unchanged during anemia (62), tissue oxygen consumption can be preserved (brain) (31), increased (heart) (42), or decreased (kidney, intestine) (30, 39, 44). These studies suggest that the degree, and threshold for, anemia-induced tissue hypoxia may be different for each organ.

At the molecular level, the importance of hypoxia-inducible factor (HIF) and hypoxia signaling has been recently demonstrated to be influenced by anemia. Severe anemia (50 g/l) activates HIF-α expression in the brain (40, 56), which occurs by S-nitrosylation of pVHL via neuronal nitric oxide (NO) synthase (nNOS)-dependent mechanisms (56). This leads to accumulation of HIF-1α expression and activation of HIF-dependent genes to promote EPO, angiogenesis (VEGF) and optimize metabolism [glucose transporter 1 (GLUT1), pyruvate dehydrogenase kinase-1 (PDK1), monocarboxylate transporter 4 (MCT) (4)] (56). In addition, other models have demonstrated the association of the HIF/hypoxia pathway with...
acute and chronic anemia (12, 17, 38). These studies emphasized the importance of HIF signaling in anemia.

Our previous studies have demonstrated tissue hypoxia and HIF activation at severe anemia (50 g/l) (56). However, higher Hb levels (>70 g/l) are usually observed in clinical settings, and the current threshold for treatment (red blood cell transfusion) is currently at or above this level (6, 8, 23). By using a transgenic mouse model in HIF-α (oxygen-dependent degradation domain, ODD) luciferase, we explore HIF expression in different organs at various Hb levels during anemia. The results of this study may have implications regarding HIF, NOS, and hypoxia signaling at clinically relevant Hb thresholds.

METHODS

Animals. All animal protocols were approved by the Animal Care and Use Committee at the Toronto Centre for Phenogenomics. HIF-α (ODD) luciferase mice were purchased (Jackson’s Laboratory), bred in-house, and maintained at the research institute. As previously described (51, 56), these mice contain a ubiquitously expressed transgene of human HIF-1α subunit (ODD region) fused with a chimeric protein containing firefly luciferase. This ODD contains a proline residue of HIF-1α that allows for hydroxylation and subsequent proteosomal degradation similar to the native HIF-1α protein.

Acute hemodilutional anemia. Spontaneously breathing HIF-α (ODD) luciferase mice were anesthetized with isoflurane (1.5%) in room air. Mice were hemodiluted in steps by exchanging equal volume of blood with pentastarch (Bristol-Myers Squibb) via the tail vein. Blood samples were collected by tail nick, and Hb concentration was measured using a hemoglobin analyzer (Hemocue). After the target Hb level (90, 70, or 50 g/l) was reached, mice were recovered from anesthesia and imaged at 6 h from the start of hemodilution (Table 1). At 6 h, animals were reanesthetized and euthanized under anesthesia, and tissues (brain, kidneys, liver) were harvested and stored at −80°C.

In vivo bioluminescent imaging. To detect luciferase expression in HIF-α (ODD)-luciferase mice, d-luciferin (50 mg/kg ip; PerkinElmer) was injected. Ten minutes later, mice were anesthetized in 1.5% isoflurane with 21% oxygen and placed in a light-tight chamber equipped with IVIS imaging camera (Xenogen 300). Dorsal and ventral images of the mouse were taken separately. Photons were collected for 10 s, and images were obtained by using LIVING IMAGE software (Xenogen) and IGOR Image analysis software. The whole body radiance represents the number of photons emitted by the body as quantitatively measured by the imaging camera (reported as photons s⁻¹ cm⁻² steradian⁻¹). These are units of photon radiance on the surface of the animal. Whole body radiance was normalized to the average from all baseline measurement (Hb 130 g/l group) and represented the fold change relative to that at baseline ~130 g/l. Images were standardized with the same color range bar for each image position.

In vitro luciferase assay. HIF-α (ODD)-luciferase mice were hemodiluted to target Hb of 90, 70, or 50 g/l and recovered for 6 h. Organs were then harvested and immediately snap frozen in liquid nitrogen. Tissues were ground to powder and underwent repeat freeze-thaw cycles in lysis buffer (Promega). Protein concentrations were determined using a BCA protein assay (Pierce). Triplicate samples were mixed with Luciferase Assay Reagent (Promega) in 96-well plates. Luciferase activity was measured by FLUOSTar Optima (BMG LABTECH). Data were normalized to protein concentration and expressed as fold change relative to baseline.

Protein measurement by Western blotting. Organs were excised from control and anemic (mild, moderate, severe) mice 6 h after hemodilution. Tissues were homogenized using a polytron (Beckman) in RIPA buffer. Protein samples were quantified, aliquoted, and stored at −80°C. Aliquots of protein from mouse tissue were separated on a SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane, and transfer efficiency was verified by Ponceau red-stained membranes. Membranes were blocked with 5% milk and probed with polyclonal HIF-1α (R&D, catalog no. AF2935) and HIF-2α (R&D, catalog no. AF2997) antibodies. Immunoblots were probed with appropriate secondary antibodies. Immunoreactive bands were detected by enhanced chemiluminescence (LG HealthScience) and quantified by densitometry using ImageJ software. Lamin (Santa Cruz, catalog no. sc-7293) or α-tubulin or (Sigma, catalog no. T-6199) were used as loading controls.

Quantitative real-time reverse transcriptase PCR. Total cellular RNA was extracted from tissue samples by a mechanical homogenizer using TRIzol (Invitrogen). First-strand cDNA was synthesized from 1 μg of RNA using random primers and Superscript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed by an ABI PRISM 7900HHT (Applied Biosystems) using a SYBR green detection system. Reactions were performed in triplicate. To quantitate copy number, serial dilution of plasmids was used corresponding to the target gene to construct a standard curve. Data were expressed in fold change after normalization to control gene 18S and to baseline.

Immunofluorescence staining. After 6 h of severe anemia (Hb ~50 g/l), HIF-α (ODD)-luciferase mice were anesthetized and perfused via the left ventricle with 4% formaldehyde. Tissues were extracted and placed in 4% formaldehyde for blocking. Immunofluorescence was performed on 10-μm thick fixed tissue sections (4% paraformaldehyde) by incubating slides overnight at 4°C with diluents of specific primary antibodies for HIF-2α (R&D, catalog no. AF2997) and luciferase (Abcam, catalog no. ab81823) to detect HIF-1α. Appropriately labeled secondary antibodies were used to detect specific bindings of the primary antibodies. Microscopy was performed utilizing fluorescent confocal microscope (Nikon ECLIPSE 90i).

Ultrasound microscopy for blood flow analysis. An ultrasound imaging system (Vevo 2100, VisualSonic, Toronto, Canada) with a 30-MHz linear array transducer was used to measure the flow at the aortic orifice, the left common carotid artery, the abdominal aorta, and the right renal artery. During ultrasound imaging, mice were anesthetized using isoflurane at 1.5% in 21% oxygen, and their body temperature was maintained at 36–37°C. The technical specifications and the procedure were described previously (65).

For measuring the aortic blood flow, the diameter of the aortic annulus was measured at peak systole in real-time two-dimensional imaging at the aortic orifice, and the Doppler velocity spectrum was recorded at the same level. Then M-mode traces and pulsed Doppler velocity spectra were recorded at the middle of the left common carotid artery, the proximal abdominal aorta, and the middle of right renal artery for measurements of flow at those locations. M-mode recording was always made with the targeted vessel perpendicular to the ultrasound beam, and the pulsed Doppler velocity was always measured with the smallest intercept angle (~60°) between the flow direction and the ultrasound beam. The imaging protocol took 30–45 min and was performed in four groups with different degrees of anemia: control (130 g/l), mild (90 g/l), moderate (70 g/l), and severe (50 g/l) (n = 6/group) and at three time points: baseline, immediately, and 6 h after hemodilution.

The intensity-weighted mean velocity of the Doppler spectrum was traced as a function of time to measure the velocity-time

Table 1. Hemoglobin concentration at the various degrees of anemia

<table>
<thead>
<tr>
<th></th>
<th>Mild (90 g/l)</th>
<th>Moderate (70 g/l)</th>
<th>Severe (50 g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>127 ± 4</td>
<td>125 ± 6</td>
<td>129 ± 5</td>
</tr>
<tr>
<td>1 h</td>
<td>90 ± 2*</td>
<td>71 ± 1*</td>
<td>52 ± 1*</td>
</tr>
<tr>
<td>6 h</td>
<td>88 ± 6*</td>
<td>86 ± 2*</td>
<td>56 ± 3*</td>
</tr>
</tbody>
</table>

Values are expressed means ± SE. *P < 0.05 vs. baseline.
integral (VTI). The flow was calculated by multiplying the VTI by the vessel area derived from the measured diameter as well as the heart rate. In addition, the peak systolic and end-diastolic velocities of the Doppler spectrum were measured for calculating the pulsatility index (PI) and resistive index (RI) for both the common carotid and the renal arteries. All parameters were averaged over three cardiac cycles.

Enzyme-linked immunoabsorbent assay. Blood from tail nick were collected into microtubes precoated with heparin. Plasma was obtained by centrifugation of blood samples for 20 min at 4°C. Enzyme-linked immunoabsorbent assay (ELISA) for erythropoietin was performed according to the manufacturer’s instruction (R&D, catalog no. MEP00B).

Statistical analysis. Statistical analysis was performed using SigmaPlot 11 (Systat). All data were normally distributed and assessed by one-way ANOVA, and post hoc analysis was performed using a Tukey test when appropriate. Data were presented as means ± SE and a value of $P < 0.05$ (two-sided denoted by * compared with values at 130 g/l; # between WT and nNOS−/−) was taken to be significant.

RESULTS

Gradual whole body HIF increase in anemia. HIF-α (ODD) luciferase mice were hemodiluted to different Hb thresholds (Table 1). Bioluminescent images demonstrated a gradual increase in whole body HIF luciferase activity as the degree of anemia became more severe (Fig. 1). The increase in HIF luciferase activity at 50 g/l is similar to our previous observations (56). The representative images demonstrated higher kidney and liver basal expression of HIF, suggesting basal tissue hypoxia in these organs. After hemodilutional anemia,
increased luciferase activity near the kidney and liver regions were observed at 6 h. However, the luciferase signal from the brain was shielded by the skull. Hence, an in vitro assay was performed in individual tissues to determine tissue luciferase activity.

**Differential organ-specific HIF1α-luciferase expression patterns in the brain, kidney, and liver.** At each Hb threshold, the pattern of HIF-1α luciferase activity at 6 h was different for the observed organs (brain, kidney, liver). At 90 g/l, HIF luciferase was reduced in the brain but remained unchanged for kidney and liver. At 70 g/l, brain HIF luciferase returned to baseline and increased in liver and remained unchanged in kidney. Finally at 50 g/l, HIF luciferase activity was increased in the brain (1.5-fold), kidney (2-fold), and liver (2-fold) (Fig. 2).

**Differential HIF-1α and HIF-2α protein expressions in the brain, kidney, and liver in anemia.** To confirm expression of organ-specific HIFα expression, Western blot analysis was performed to assess HIF-1α and HIF-2α expression (Fig. 3). The results demonstrated a similar pattern for HIF-1α protein in the brain (reduced at 90 g/l, returned to baseline at 70 g/l, and increased at 50 g/l). However, HIF-2α did not increase at any anemia level. These specific HIF-1α responses, without a HIF-2α response in the brain, are consistent with previously reported values at severe anemia (Hb 50 g/l) (56). By contrast, in the kidney, both HIF-1α and HIF-2α levels were increased at all levels of anemia suggesting a different (hypoxic) mechanism for HIF-α stabilization. In the liver, the HIF-1α level was increased at 70 g/l and robustly increased to threefold at 50 g/l, whereas HIF-2α was increased by 1.5-fold at 90 g/l and remained at
this level to 50 g/l. Representative blots are shown in Fig. 3 (n = 5).

Immunofluorescence studies, using an antibody to luciferase, demonstrated that HIF-1α luciferase is expressed in the perivascular regions in brain section during severe anemia while HIF-2α is not increased (Fig. 4). In the kidneys, HIF-2α was heavily expressed in the tubular regions relative to HIF-1α (Fig. 4).

Differential HIF-dependent genes in the brain and kidney. HIF-dependent RNA levels were assessed in the brain and kidney at different Hb thresholds. Some genes (PDK1, GLUT1) tended to increase gradually and demonstrated similar response patterns in the two organs. Conversely, the expression patterns of other genes were different in brain and kidney (MCT4, EPO). While MCT4 and EPO levels were increased in both the brain and kidney at all levels of anemia, the increase was much greater in kidneys relative to the brain. The fold changes of MCT4 and EPO were higher in the kidney at all levels of anemia compared with the brain, suggesting that HIF-dependent genes respond more profoundly and at a higher Hb threshold in the kidneys (Fig. 5).

NOS RNA levels are differentially regulated in the brain and kidney. We previously demonstrated that NO can regulate brain HIF-1α expression in severe anemia (56). We sought to determine how NOS enzymes behave in the brain and kidney at the various Hb thresholds. In the brain, nNOS increased twofold at mild and moderate anemia, followed by a smaller increase (1.5-fold) at severe anemia. In contrast, brain endothelial NOS (eNOS) expression did not change in any degree of anemia. The increase in brain nNOS and stable eNOS levels in anemia are consistent with previous reports (22, 40, 56). In contrast to the brain, both nNOS and eNOS expressions were increased (2.0-fold and 1.5-fold, respectively) at all levels of anemia in the kidneys (Fig. 6).

Increased HIF luciferase in the kidney is independent of nNOS. We have previously reported that HIF luciferase is increased in the whole body and the brain of anemic nNOS-replete mice, but not in nNOS-deficient mice, suggesting nNOS is critically important in stabilizing HIF-1α in whole body and the brain (Fig. 7) (56). By contrast, HIF luciferase activity increased even further in anemic nNOS-deficient kidneys, suggesting mechanisms other than nNOS are responsible for kidney HIF-1α stabilization (Fig. 7). As in the brain, liver HIF luciferase data demonstrate an increase in nNOS-replete but not nNOS-deficient mice.

Differential carotid and renal blood flow in brain and kidney. Measurements of aortic, carotid, and renal blood flow demonstrate that the progressive increase in cardiac output associated with acute anemia is disproportionately delivered to brain at the expense of renal blood flow (Fig. 8; Table 2). An increase in ascending aorta and carotid blood flow immediately after hemodilution (1 h) occurs at 90 g/l and remained high until 50 g/l. This early increase in aortic and carotid blood flow is consistent with our previous early cardiac output increase observed at 90 to 50 g/l (56). Abdominal aortic blood flow did not increase until at Hb threshold of 50 g/l. This is associated with a late and small increase in renal blood flow at 50 g/l. These changes in blood flow returned toward baseline levels 6 h after hemodilution.

Plasma EPO increased at a Hb threshold near 100 g/l. Plasma EPO levels at 6 h increased significantly and progressively in all levels of anemia (Fig. 9). A scatter plot of EPO and Hb levels suggests that an increase in EPO occurs near Hb of 90 g/l. This may suggest that the kidney senses tissue hypoxia early at a high Hb threshold near 90 g/l.
DISCUSSION

The primary results of this study demonstrated that the brain, kidney, and liver behave very differently at various Hb levels in anemia. By assessing the following outcomes: 1) HIF-luciferase activity, 2) HIF-1α and HIF-2α protein expression, and 3) HIF-dependent RNA genes, we observed the novel finding that different organs have variable expression of hypoxic cellular responses to mild (90 g/l) and moderate (70 g/l) anemia. These data demonstrate heterogeneity of the HIF responses at clinically relevant Hb thresholds. We confirmed the HIF responses at severe anemia (50 g/l) reported in our earlier work (40, 56). Importantly, HIF-1α and HIF-2α appeared to be differentially regulated in the brain, which demonstrated an nNOS-dependent HIF-1α response, but no HIF-2α response. In the kidneys, both HIF-1α and HIF-2α expression levels are comparably increased in an nNOS-independent manner. Blood flow data confirm that preferential perfusion of the brain is maintained by increasing carotid blood flow at all levels of anemia, whereas renal blood flow is not increased until very low Hb values are reached. Preferential perfusion of the brain may explain why a novel “nonhypoxic” mechanism is utilized for HIF-1α stabilization (i.e., nNOS), while relatively severe tissue hypoxia appears to enhance both HIF-1 and 2α stabilization in the kidney. The HIF response in the liver appears to be predominantly due to an increase in HIF-1α and may also be nNOS dependent.

This study also demonstrated that the Hb threshold for increased systemic EPO is near 90 g/l, close to the level where increased cerebral and renal injuries are observed in the perioperative settings (36). While the mechanism of anemia-induced organ injury remains incompletely defined, tissue hypoxia is believed to be a main contributor. In addition, animal
studies have demonstrated that acute hemodilutional anemia leads to tissue hypoxia, or inadequate organ perfusion, despite increases in blood flow to vital organs such as the brain, kidney, and liver (27, 30, 39, 44, 48, 56–58). At the cellular level, demonstration of increased hypoxia markers, such as HIF, EPO, and VEGF, may represent activation of adaptive responses to tissue hypoxia (40). Our previous work suggests that nNOS-dependent expression of HIF-α may support survival during anemia (56). The current data supports the view that oxygen-dependent but nNOS-independent activation of HIF in the kidney may be an early warning signal for anemia-induced tissue hypoxia.

The heterogeneous HIF responses in the brain, kidney, and liver may reflect differential roles of these vital organs during anemia. The brain requires maintained preferential oxygen delivery due to its central regulatory role and high oxygen...
levels, and reaches a lower tissue PO2 (48, 57). This occurs in anemia the kidney becomes hypoxic earlier, at higher Hb levels. Remote sentinel signal generated in response to anemia-induced adaptive responses (HIF) without a precipitous drop in oxygen mechanisms (nNOS), especially in the brain, which maintains preferential cerebral vasodilatation, which direct a high proportion of the cardiac output toward the brain; 2) an increase in oxygen extraction from Hb and possibly a reduction in brain metabolism; and 3) early upregulation of additional signaling mechanisms (nNOS), especially in the brain, which maintains adaptive responses (HIF) without a precipitous drop in oxygen delivery. These brain responses may be activated by an earlier, remote sentinel signal generated in response to anemia-induced tissue hypoxia. The kidney is one obvious candidate: during anemia the kidney becomes hypoxic earlier, at higher Hb levels, and reaches a lower tissue PO2 (48, 57). This occurs despite a measured increase in cortical and medullary microvascular blood flow (10, 30). In our model, we demonstrated a lack of renal artery blood flow in response to acute anemia relative to the carotid artery, supporting the argument that the mechanism to optimize oxygen delivery to the kidney may be inadequate during acute anemia. In addition, other investigators have demonstrated that a diffusive shunting mechanism exists within the kidney, which appears to be differentially affected by factors such as blood flow, Hb concentration, and arterial PO2 (16). These mechanisms are potentially responsible for the enhanced renal tissue hypoxia in anemia, as is evident from the kidney’s protein and RNA response and the early increase in systemic plasma EPO levels. Thus the kidney provides a vital hypoxia-sensing role to activate the adaptive systemic physiological responses to anemia.

Local tissue responses might help explain the differential HIF-1α and -2α expression in the brain, kidney, and liver. The brain and liver appear to have predominantly HIF-1α responses. In the brain, a rapid and disproportional increase in blood flow may increase cerebral oxygen delivery and reduce HIF expression (Hb of 90 g/l) as demonstrated by our data. Additional mechanisms, including the increase in oxygen extraction during anemia (48, 58), may augment oxygen delivery and contribute to the observed early drop in HIF-1α levels. However, as Hb levels and brain oxygen delivery are further reduced, the increase in brain tissue blood flow and oxygen extraction cannot adequately maintain tissue oxygen tension (56), resulting in the delayed reduction in brain PO2, which corresponds to a late rise in HIF-1α. This is only observed during severe anemia at Hb values near 50 g/l. This brain HIF-1α response is dependent on nNOS and is not accompanied by an increase in HIF-2α levels. By contrast, the kidney demonstrates an early decrease in tissue PO2 and a limited or inadequate increase in renal blood flow. These responses result in a more severe degree of tissue hypoxia as reflected by the earlier combined HIF-1α and HIF-2α protein response. Furthermore, the degree of the hypoxic response to anemia in the kidney is reflected by the extremely high HIF-dependent RNA responses, particularly for EPO (HIF-2α response) (30). This imbalance in tissue oxygen supply and demand is likely much higher in the renal medulla, which performs a larger degree of metabolic work, relative to its blood flow (10), and the effect of the counter current mechanism, which is known to contribute to the decreased medullary oxygen content. These data suggest that even a slight decrease in Hb may result in inadequate renal oxygen delivery and impaired renal oxygen homeostasis. Unlike the brain, this abrupt fall in kidney PO2 at relatively high Hb (48, 57) results in an early increase in HIF-1α and HIF-2α signaling. These responses are augmented in the nNOS-deficient mice and therefore are likely not dependent on protein nitrosylation (Fig. 7). A third pattern of HIF expression occurs in the liver; the HIF response is predomi-
nanty HIF-1α and this response appears to be attenuated in the absence of nNOS, as reflected by the lack of HIF-1α response in nNOS-deficient mice.

Despite the fact that HIF-1α and HIF-2α both contribute to the hypoxia response pathway and are structurally similar, accumulative evidence has demonstrated that they are different in many ways. From the perspective of tissue specificity, HIF-1α is ubiquitously expressed, whereas HIF-2α expression is relatively restricted to several tissues such as the endothelium, kidney, lung, and heart (13, 55, 63). Within the tissue, cell type specificity is also found between the two HIF-α isoforms. For example, in the brain, HIF-1α is mainly found in neurons (2), whereas HIF-2α is expressed in astrocytes (9). In the kidney, tubular cells express HIF-1α, whereas endothelial cells and fibroblasts express HIF-2α (50). Structurally, the difference in their NH2-terminal transactivation domains is important for target gene specificity of HIF-1α and HIF-2α (25). HIF-1α is primarily responsible for regulation of glycolytic enzymes (26), whereas the in vivo EPO response is predominately regulated by HIF-2α (32, 45, 49). Strong evidence of predominant HIF-2α expression is seen in the renal production of EPO during acute anemia.

The mechanisms of increased HIF-1α expression appear to be different between the brain and kidney. As a master regulator of genes involved in the adaptation to hypoxia, HIF-α protein expression can be controlled by both oxygen-dependent and -independent mechanisms. During hypoxia, HIF-α protein is stabilized by escaping the degradation pathway via inactivation of the prolyl hydroxylases enzymes. In anemia, HIF-α protein can be stabilized by nNOS-derived NO and S-nitrosylation of pVHL despite the presence of oxygen (56), thereby inhibiting the degradation pathway. The dependency of HIF-1α expression on nNOS occurs specifically in the brain perivascular regions, despite a small reduction in brain tissue PO2. By contrast, the increase in kidney HIF-1α could be predominantly due to tissue hypoxia rather than nNOS. This is supported by the observation that a larger HIF luciferase activity is observed in the kidneys of nNOS-deficient mice relative to nNOS-replete mice at severe anemia. Despite the finding that anemic nNOS-deficient mice died earlier and lacked cardiac output response, their carotid blood flow and brain PO2 is comparable to that of the survived nNOS-replete mice (56). This implies that brain blood flow and oxygen delivery was preferentially maintained at the expense of other organ blood flow (e.g., kidneys), which did not increase. These perfusion differences may have contributed to the observed severe hypoxic response in the kidneys. Although we did not directly measure kidney tissue PO2, our lab and others have reported a much larger decreases in kidney PO2 during anemia relative to the brain (30, 48, 57, 58). Importantly, biochemical studies revealed that nNOS enzymatic activity depends heavily on oxygen (Km for O2 of 350 μM) (53). This suggests that “higher” PO2 is required to increase nNOS activity, such as in the brain, to stabilize HIF-1α. However, “lower” PO2 in kidney may inhibit nNOS activity. Thus kidney HIF-1α can be stabilized by hypoxia via the classical prolyl hydroxylase domain

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (130 g/l)</th>
<th>Mild (90 g/l)</th>
<th>Moderate (70 g/l)</th>
<th>Severe (50 g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 1 h 6 h</td>
<td>Baseline 1 h 6 h</td>
<td>Baseline 1 h 6 h</td>
<td>Baseline 1 h 6 h</td>
</tr>
<tr>
<td>Blood flow, ml/min</td>
<td>7.04 ± 0.38</td>
<td>7.12 ± 0.44</td>
<td>7.22 ± 0.28</td>
<td>7.04 ± 0.51</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>369 ± 13</td>
<td>447 ± 13*</td>
<td>421 ± 12*</td>
<td>414 ± 18</td>
</tr>
<tr>
<td>VTI, mm 20.6</td>
<td>118.3 ± 1.4</td>
<td>17.5 ± 0.6*</td>
<td>20.1 ± 0.9</td>
<td>17.4 ± 1.5</td>
</tr>
<tr>
<td>AO diameter, mm</td>
<td>1.06 ± 0.02</td>
<td>1.08 ± 0.01</td>
<td>1.05 ± 0.01</td>
<td>1.12 ± 0.02</td>
</tr>
<tr>
<td>Blood flow, ml/min</td>
<td>0.71 ± 0.03</td>
<td>0.81 ± 0.1</td>
<td>0.78 ± 0.08</td>
<td>0.92 ± 0.13</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>371 ± 10</td>
<td>413 ± 5*</td>
<td>405 ± 9*</td>
<td>397 ± 22</td>
</tr>
<tr>
<td>VTI, mm 20.6</td>
<td>11.8 ± 0.3</td>
<td>13.2 ± 1.4</td>
<td>13.4 ± 1.1</td>
<td>14.0 ± 2.1</td>
</tr>
<tr>
<td>Diameter, mm</td>
<td>0.45 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>RI</td>
<td>0.78 ± 0.01</td>
<td>0.75 ± 0.01</td>
<td>0.76 ± 0.01</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td>Blood flow, ml/min</td>
<td>5.87 ± 0.53</td>
<td>6.17 ± 0.95</td>
<td>6.75 ± 0.83</td>
<td>6.48 ± 0.47</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>343 ± 14</td>
<td>384 ± 15*</td>
<td>371 ± 8</td>
<td>382 ± 22</td>
</tr>
<tr>
<td>VTI, mm 20.6</td>
<td>20.6 ± 1.4</td>
<td>19.6 ± 2.0</td>
<td>20.7 ± 1.8</td>
<td>19.2 ± 1.7</td>
</tr>
<tr>
<td>Diameter, mm</td>
<td>1.03 ± 0.02</td>
<td>1.01 ± 0.03</td>
<td>1.03 ± 0.04</td>
<td>1.03 ± 0.03</td>
</tr>
<tr>
<td>Blood flow, ml/min</td>
<td>0.91 ± 0.08</td>
<td>0.79 ± 0.09</td>
<td>0.69 ± 0.10</td>
<td>0.83 ± 0.10</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>367 ± 15</td>
<td>379 ± 12</td>
<td>385 ± 7</td>
<td>374 ± 16</td>
</tr>
<tr>
<td>VTI, mm 20.6</td>
<td>21.3 ± 2.4</td>
<td>19.7 ± 1.9</td>
<td>18.3 ± 2.4</td>
<td>21.3 ± 3.6</td>
</tr>
<tr>
<td>Diameter, mm</td>
<td>0.39 ± 0.01</td>
<td>0.37 ± 0.02</td>
<td>0.35 ± 0.01</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td>RI</td>
<td>1.48 ± 1.3</td>
<td>14.9 ± 2.2</td>
<td>16.1 ± 2.3</td>
<td>12.9 ± 1.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. AO, aortic oriﬁce; PI, pulsatility index; RI, resistive index; VTI, velocity-time integral of Doppler flow waveform. *P < 0.05 vs. baseline.
decrease in this hypoxic environment (14, 15, 61). Increased eNOS expression in hypoxia, especially in the vascular endothelium decreases in eNOS transcription, mRNA stability, and protein expression. Work from us and others have described the ed rational mechanisms by which HIF-1α/HIF-1β/PHD pathway. These results highlight the different mechanisms by which HIF-1α can be regulated based on the local tissue oxygen environment.

In addition to the varying HIF responses in the brain and kidney, the disparity also occurs at the level of NOS enzyme regulation. Our earlier work had consistently documented an increase in nNOS and stable eNOS expression in the brain of anemic animals (22, 40, 56). In the brain, anemia-induced nNOS expression may represent an adaptive response necessary for improving cerebral blood flow (28) and modulating protein function via S-nitrosylation (29). In the kidneys, nNOS regulates tubuloglomerular feedback in the macula densa (64) and controls electrolyte and fluid balance in proximal tubules and the collecting duct (59, 60). The increase in nNOS expression in anemia mirrors that of hypoxia in the brain, kidney, aorta, and vascular smooth muscle (61). In hypoxia, a shortened nNOS mRNA transcript is produced, leading to efficient protein translation that is otherwise very inefficient in the basal state. A similar mechanism may serve to increase nNOS in anemia. To our surprise, anemia led to an increase in kidney eNOS expression. Work from us and others have described decreases in eNOS transcription, mRNA stability, and protein expression in hypoxia, especially in the vascular endothelium (14, 15, 24, 41). We would have anticipated that eNOS would decrease in this hypoxic environment (14, 15, 61). Increased renal eNOS expression may help improve renal blood flow during anemia. This paradoxical eNOS finding reinforced the notion that anemia and hypoxia are distinct (56). Further work will be required to elucidate the function of these enzymes in kidneys during anemia. These data again highlight the unappreciated cellular differences in the brain and kidneys.

Whereas changes in blood viscosity have likely influenced tissue oxygen delivery in our model (5), we suggest that other mechanisms which actively regulate cardiac output and tissuespecific vascular resistance likely contribute based on the following lines of reasoning: 1) changes in blood viscosity would be common to all vascular beds, whereas the differences in organ-specific blood flow and oxygen delivery may be due to differential viscosity sensing in the microvascular beds, the authors favor the argument that local sensing of tissue hypoxia is likely the primary contributing mechanism as organs with the highest oxygen requirement receive the highest degree of increased blood flow; 2) similar degrees of hemodilution and viscosity reduction were achieved in nNOS-replete and -deficient mice (56); however, this resulted in dramatically different cardiovascular responses and oxygen delivery patterns in between these two strains, suggesting that mechanisms other than rheology were responsible; and 3) active inhibition of both cardiac output and active microvascular dilation during acute hemodilution greatly impaired brain tissue oxygen delivery by mechanisms independent of changes in blood viscosity, which were controlled for in these experimental protocols (21, 27, 48).

Our results are comparable to that of Lauscher et al. (37), which demonstrated heterogeneity of tissue hypoxia during severe anemia in a porcine model. Utilizing pimonidazole tissue binding methodology, they demonstrated evidence of tissue hypoxia (PO2 <10 mmHg) in the heart, brain, kidney, and liver at Hb levels below 40 g/l. Our data differs from this study in that we utilized real-time assessment of HIF responses that are more sensitive to subtle changes of PO2 within the physiological range. We and others have observed early oxygen sensing at Hb values as high as 90 g/l. The physiological and cellular responses to acute reduction in Hb occur at a relatively high Hb threshold and in proportion to the change in Hb (i.e., tissue O2 delivery). This suggests the existence of complex and integrated ability of cells to detect tissue hypoxia and activate adaptive physiological responses to preserve oxygen homeostasis. From a clinical perspective, identifying an early threshold for anemia-induced tissue hypoxia may explain why acute anemia is associated with renal and brain injury at Hb levels near 70 g/l (33, 35, 36). Indeed, measuring changes in tissue PO2 at excessively low Hb values (40 g/l) near the critical Hb level (<30 g/l) likely has little clinical relevance; as clinical treatment threshold are well above this value and evidence of tissue injury occurs much at much higher Hb concentrations. We hope to utilize evidence of early anemia-induced tissue hypoxia to develop biomarkers to guide clinical treatment of acute anemia to improve event-free survival in patients undergoing surgery.

There are limitations in our work. We have not directly measured tissue PO2 at 6 h after hemodilution to match the protein and RNA expression. Also, the cellular responses to different Hb thresholds in chronic anemia need to be addressed. As we have previously observed, there was a rebound increase in Hb after 6 h (56). This effect was most pronounced.

**Fig. 9.** Plasma erythropoietin (EPO) increased at mild anemia near hemoglobin threshold of 90 g/l. A correlation of systemic EPO and hemoglobin concentration demonstrated increased systemic EPO below 90 g/l 6 h following hemodilution. EPO levels increased progressively and proportionally to the degree of anemia. *p < 0.05 vs. hemoglobin threshold 130 g/l. One-way ANOVA analysis was used.
in the moderate group but also observed in the severely anemic group and may have influenced O₂ delivery and HIF measurements. In addition, a more comprehensive cellular profile on other vital organs, such as the heart and liver, will be of interest. Additionally, the mechanism by which HIF-1α decreases at mild anemia will need to be defined in future studies. The issue of starch colloid and its effects on renal function is controversial. In three clinical trials (VISEP, CHEST, 6S), extended use of relatively high dose of starch colloid administer over days negatively affected renal function in patients with sepsis (4, 43, 46). In one additional trial (CHRYSMAS), no adverse effect of colloid on renal function was identified (18). Despite this controversy, we acknowledge that pentastarch may have impacted renal function in our experimental protocol. However, our data support an independent effect of anemia (not starch colloid) on renal and other tissue HIF expression for the following reasons: 1) we found no evidence of renal failure in our rodent model of acute anemia in which animals were exposed to only 1 dose of colloid within 7 days (56); and 2) controlled experiments in transgenic and β-blocked rodents demonstrated an accentuated hypoxic response when cardiovascular mechanisms were impaired. These effects were independent of colloid, since the effect of colloid exposure was controlled for in the experimental groups (27, 48, 56). Therefore, the heterogeneous tissue-specific HIF responses that we have observed were due to the degree of anemia and not likely to be secondary to a direct colloid effect.

In summary, the findings of this study demonstrate an organ-specific Hb threshold for increased HIF-α stabilization. The mechanism by which HIF-1α increased in brain and kidney appear to be different (nNOS vs. hypoxia). These differential cellular patterns may be due to the local oxygen delivery and extraction characteristics. This knowledge may help define the mechanism(s) by which the brain and kidney maintain oxygen homeostasis during anemia. The data may also help to identify potential mechanisms of anemia-induced organ injury when these adaptive responses are overwhelmed and may help to develop novel treatment strategies to reduce morbidity and mortality in anemic patients.

**Perspectives and Significance**

These data continue to support the observation that the adaptive responses to acute anemia result in heterogeneous organ-specific hypoxic tissue responses. The data indicate that redundant mechanisms act to maintain oxygen homeostasis in the brain (increased cardiac output, preferential carotid blood flow, nNOS). The kidney may experience increased tissue hypoxia due to a lack of these mechanisms. This may support the hypothesis that the kidney may be a central hypoxia-sensing organ that triggers adaptive systemic responses to acute anemia at a relatively high Hb threshold. The clinical correlate that acute anemia is associated with acute renal failure (33, 54) may support this argument and may help to direct translational research to help define patient-specific biomarkers of tissue hypoxia that can be used to direct therapy.

**GRANTS**

Support was provided by the Society of Cardiovascular Anesthesiologists-IARS Mid-Career Grants (to G. M. T. Hare); Canadian Anesthesiologist’s Society (to G. M. T. Hare); Department of Anesthesia, University of Toronto Merit Awards (to G. M. T. Hare, C.D. Mazer); Heart and Stroke Foundation T7172 (to P. A. Marsden).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00411.2013 • www.ajpregu.org
R24

ORGAN-SPECIFIC HIF AND NOS RESPONSES TO ANEMIA


