Immunohistochemical Localization of Hepatic Nitric Oxide Synthase in Normal and Transgenic Sickle Cell Mice: The Effect of Hypoxia

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Nitric oxide (NO) generated from L-arginine and molecular oxygen by nitric oxide synthase (NOS) has been shown to influence hepatocellular function and pathology in response to ischemia and certain hepatotoxins. In the present study, we examined the liver of a transgenic line of sickle cell mice for hepatocellular injury and localization of two isoforms of NOS, the endothelial constitutively expressed isoform (EcNOS) and the inducible isoform (iNOS) by immunohistochemistry. Diffuse expression of EcNOS was observed in hepatocytes of control and sickle cell animals maintained under room air conditions. In contrast, iNOS was observed only in the sickle cell mice, well-localized to hepatocytes surrounding the central veins of the lobules. When normal mice were exposed to hypoxic conditions for 4 to 5 days, iNOS immunostaining appeared de novo in a patchy distribution throughout the liver lobules. In the sickle cell mice, hypoxia appeared to increase the subjective intensity of pericentral staining of iNOS. Liver histology was normal in the sickle cell mice maintained under room air conditions, but showed multifocal areas of necrosis when sickling was exacerbated by chronic hypoxic conditions. However, a pericentral zone of preserved architecture was present, corresponding to the region of iNOS staining. We postulate that pericentral induction of iNOS under ambient conditions occurs in transgenic sickle cell mice in response to particularly intense hypoxic conditions near the central veins of the liver. Increases in NO synthesis may occur in this region, which would serve to protect these cells from ischemic damage either directly or by maintaining blood flow. These findings could be relevant to liver pathophysiology in patients with sickle cell disease.

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HEPATIC DYSFUNCTION in patients with sickle cell disease is well-recognized and can result from viral hepatitis, extrahepatic or intrahepatic cholestasis, or sickle cell vasooclusion that may be either acute or chronic. Hepatic sickle cell crisis is an acute, transient syndrome consisting of intrahepatic bilary obstruction presenting with acute right upper quadrant pain, hepatic enlargement, fever, and jaundice. It runs a short clinical course, often subsiding spontaneously within 3 to 7 days, and is often seen in combination with other clinical and hematological features of sickle cell painful crisis. Hepatomegaly is present in 40% to 80% of patients in clinical series, and 80% to 100% in autopsy series. Most enlarged livers in sickle cell patients are associated with histopathological findings of dilated sinusoids and Kupffer cell erythrophagocytosis. Other less common findings include hemosiderosis, focal necrosis, nodular hyperplasia, portal fibrosis, and micronodular cirrhosis. The mechanism of acute and chronic hepatic cellular injury in sickle cell disease is unclear, but tissue hypoxia resulting from repeated episodes of vasooclusion, Kupffer cell erythrophagocytosis, and local sinusoidal obstruction have been postulated to cause cellular injury and subsequent repair fibrosis. Sinusoidal obstruction is not surprising, as low rates of blood flow in the sinusoids are likely to be particularly detrimental to sickle cells, due to low PO2 and pH, which enhance sickling. The generation of nitric oxide (NO) from L-arginine and molecular oxygen has been proposed to either mediate or modulate cellular damage in several organs including the liver, kidney, and brain. NO has been implicated in hepatocellular injury following ischemia-reperfusion and endotoxina. Three isoforms of nitric oxide synthase (NOS) have been described. A neuronal constitutive isoform (NOS I or bNOS) is found primarily in neurons in the central and peripheral nervous system. An endothelial-derived constitutive isoform (EcNOS or NOS III) is expressed by endothelial cells and several other organs including neurons, kidney, and liver. A cytokine/endotoxin inducible isoform (iNOS or NOS II) is expressed by macrophages, leukocytes, glia, and many organs in response to infections, cytokines, mechanical injury, and hypoxia. The recent availability of specific antibodies directed against NOS isoforms has facilitated understanding of their cellular distribution and how that may impact the pathogenesis of liver dysfunction. For example, in an endotoxemia model of liver damage, iNOS immunostaining appeared in Kupffer cells, other macrophages, and leukocytes. The increased production of NO that might occur as a result of activation of iNOS has been suggested to reduce hepatic damage, to increase hepatic damage or to enter into a reaction with superoxide radicals the balance of which determines cellular toxicity. In sickle cell disease, liver damage may also be affected by changes in local production of NO. To study this question, we performed an immunohistochemistry examination of the liver in a transgenic mouse model of sickle cell disease, α(αβ)MDD. The cellular distribution of two isoforms of NOS, EcNOS and iNOS, were examined in livers of control and α(αβ)MDD mice maintained under either room air or chronic hypoxic conditions; the latter enhances endogenous HbS polymerization and in vivo sickling.

MATERIALS AND METHODS

Animals and tissue preparation. The methods for creating the transgenic sickle cell α(αβ)MDD mice and their characteristics have been described in previous publications. Briefly, this line of transgenic mice was created by the simultaneous microinjection of α(αβ)MDD into fertilized eggs from transgenic HbS/pMDD mice. The transgenic line was obtained by microinjection into fertilized eggs from transgenic mice. The transgenic sickle cell mice (aHpS[pMDD]) were maintained under either room air or chronic hypoxic conditions.

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two constructs each containing the locus control region (LCR) of the human β-globin gene and either the α2-gene or β0 globin gene into fertilized eggs of the friend virus strain B (FVB) mouse. The mice with stable cointegration of αβ and β0 were then bred onto a C57Bl/6J background for seven or more generations. Higher levels of human β0 were achieved by breeding the transgenic mice with C57Bl mice bearing a spontaneous deletion of the mouse β major-globin gene. When the β0 insertion was bred to homozygosity, expression of β0 averaged 72.7%. These mice are designated as αHps[PMDD] indicating a homozygous deletion of mouse β0 globin. Normal mice of the strain C57Bl/6J were purchased from Jackson Laboratories (Bar Harbor, ME). The mice (n = 4 per treatment group) were housed in individual cages under room air or in glass environmental chambers (Braintree Scientific, Braintree, MA) filled with constantly flowing 10% O2/0.5% CO2/89.5% N2 gas for 4 to 5 days. Animals were allowed free access to water and mouse pellet diet up to the day they were killed. The mice were anesthetized, and transcardiac perfusion was performed with cold phosphate-buffered saline (PBS) pH 7.6 for 10 minutes followed by perfusion fixation with paraformaldehyde-lysine-periodate for 10 minutes. Livers were dissected, blocks of tissue taken from similar regions, postfixed in the same fixative for 12 hours at room temperature, and then postfixed for embedding in paraplast. Serial four micron-thick sections were cut on a microtome and stored until use.

**Immunohistochemistry and microscopy.** Tissue sections selected from similar lobes of the liver of the four groups of mice were selected and processed simultaneously on the same day for immunostaining of NOS isoforms. Because all reagents and timing of staining was the same in the four groups, a qualitative comparison of staining intensity could be made. The specimens were dehydrated twice in xylene for 10 minutes each, rehydrated in ethanol, and washed in distilled water for 1 minute and PBS for 10 minutes. Endogenous peroxidase was blocked by immersion in 0.03% hydrogen peroxide in methanol for 20 minutes and washed twice in PBS for 10 minutes each. Nonspecific binding was blocked and sections were permeabilized with a mixture of 0.3% Triton X-100, 0.2% normal goat serum, and 0.5% bovine serum albumin in PBS (PBA) for 20 minutes. After blotting to remove excess serum, tissue sections were then incubated with PBA containing antiserum to NOS isoforms (Transduction Labs, Lexington, KY). These antisera were generated against specific peptide sequences of NOS isoforms, and their tissue specificity has been determined by previous studies in our laboratory.1 In preliminary experiments the presence of immunostaining for iNOS and EcNOS in the liver; nNOS was not observed in the liver. Therefore, for the study, tissue sections were incubated with either anti-iNOS or anti-EcNOS at an optimum dilution of 16 µg/mL for 1 hour at room temperature. Adjacent sections, which served as controls, were incubated with preimmune serum, or the primary antibody was omitted in the staining procedure. After washing the sections twice in PBS for 5 minutes each, NOS immunostaining was detected with the Vectastain peroxidase kit (Vector Labs, Burlingame, CA). Optimum color reaction was developed with diaminobenzidine tetrahydrochloride for 5 minutes. Sections were then counterstained with hematoxylin, rehydrated with ethanol, cleared with xylene and coverslipped. For routine histological analysis, adjacent sections were stained with hematoxylin-eosin (H&E). Sections from similar lobes of normal and αHps[PMDD] mice housed under room air were compared with sections from mice exposed to chronic hypoxia. The sections were coded and analyzed by two observers blinded to the results with a Nikon Optiphot microscope equipped with a Nikon FX35A camera (Yokohama, Japan). The following parameters were evaluated: iNOS and EcNOS immunostaining within zones of the hepatic lobule, ie, in relation to the central veins and portal areas, and intracellular location. The relative intensity of immunostaining within cells was also scored as absent, similar, or increased in comparison to coded standards from normal and αHps[PMDD] mice housed under room air and processed simultaneously under the same conditions.

**Aspartate amino transferase.** Plasma samples were obtained from C57Bl/6J, and αHps[PMDD] mice by collecting blood from a tail incision in heparinized microhemocrit tubes. The samples were centrifuged to obtain the plasma. They were analyzed using the Technicon Chem-1 system (Technicon, Tarrytown, NY) and results for aspartate amino transferase were expressed in units/mL. Samples were collected under both ambient and chronic hypoxic conditions, using the same protocol as for the histology studies.

**RESULTS**

**Histology.** H&E-stained liver sections of control and αHps[PMDD] mice maintained under room air showed normal histology. In contrast, under chronic hypoxic conditions, the liver of the αHps[PMDD] mice manifested multifocal infarcts characterized by coagulative necrosis, hepatocytes lacking nuclei, and loss of outlines of hepatic plates and dilated sinusoids. A typical example is shown in Fig 1A. Normal mice maintained under hypoxic conditions did not show any histological abnormalities.

**Immunohistochemistry.** Control sections of the liver incubated with preimmune serum or absent the primary antisera did not show specific staining. In animals housed under room air conditions, immunohistochemistry of the liver showed a diffuse distribution of EcNOS immunostaining throughout the hepatic lobules of both normal (Fig 1B) and αHps[PMDD] mice. There was no change in the pattern of EcNOS staining in either normal or αHps[PMDD] mice when they were exposed to chronic hypoxia. iNOS immunoreactivity was absent in liver sections of normal mice housed under room air conditions (Fig 1C). However, when normal mice were exposed to chronic hypoxia, iNOS immunostaining was observed scattered throughout the hepatic lobules (Fig 1D). In the αHps[PMDD] transgenic mice, iNOS immunostaining was present even under room air conditions, and was sharply localized to pericentral hepatocytes (Fig 1D). This zonal distribution of iNOS immunostaining persisted after exposure to chronic hypoxia, although the relative intensity of staining appeared to be increased (compare Fig 1E with 1F).

**Aspartate amino transferase.** In control (C57Bl/6J) mice maintained under room air conditions, the level of aspartate amino transferase was 51.2 ± 3.4 U/mL (mean ± SE, n = 10). In the αHps[PMDD] mice, the level was 61.0 ± 4.3 (n = 20). Although the mean value was higher in the αHps[PMDD] mice than in the controls, the difference was not statistically significant. When two C57Bl/6J mice were exposed to hypoxia for 5 days, there was no apparent change in plasma amino transferase levels. In contrast, when αHps[PMDD] mice were subjected to hypoxia for 5 days, their plasma level of aspartate amino transferase rose to an average value of 125.7 ± 9.3 U/mL, respectively (Fig 2). This value is statistically different from that found for the αHps[PMDD] mice under room air (P < .00002). These elevated enzyme levels presumably relate to the abnormal histology found in separate αHps[PMDD] mice exposed to hypoxia (Fig 1A).
**Fig 1.** (A) Hematoxylin and eosin-stained section of liver from transgenic sickle cell mouse reared under chronic hypoxia. Note area of infarct, loss of outline of hepatic plate, and dilated sinusoids. (B) Diffuse EcNOS-immunostaining of hepatocytes in a normal mouse under room air. (C) Absence of iNOS in liver of a normal mouse under room air. The background staining was similar to adjacent control sections incubated with preimmune serum or when primary antisera were omitted. (D) iNOS immunostaining in scattered hepatocytes in a normal mouse exposed to chronic hypoxia (arrows). (E) Moderate iNOS-immunostaining in pericentral hepatocytes of \( \alpha\beta[\beta^{-\text{n}}] \) mouse under room air. (F) Increase in pericentral iNOS in transgenic mouse exposed to hypoxia. Scale bar = 100 \( \mu \)m in A, C, D; 50 \( \mu \)m in B, E, F.
patchy liver necrosis and elevation of plasma aspartate amino transferase concentrations when exposed to a low oxygen environment. The hepatocellular damage is presumed to be caused by vasoocclusion-mediated tissue hypoxia resulting from Hbs polymerization and sickling. These results are consistent with a previous study performed in a different strain of transgenic sickle cell mice, a strain expressing both constitutive NOS (EcNOS) and inducible NOS (iNOS). We found differences in the expression of these two isoforms of NOS in hepatocytes of normal versus transgenic sickle cell mice. EcNOS was seen diffusely in hepatocytes of both groups of mice maintained under room air conditions, and was not affected by exposure to 10% O2 for 4 to 5 days. In contrast, iNOS staining was not observed at all in the normal mice, but was strongly positive in the hepatocytes of α H(βK)βMDD mice. In these animals, iNOS was sharply localized to a zone surrounding the central veins of the liver lobules. Exposure of control mice to hypoxia induced iNOS staining in hepatocytes scattered throughout the lobules. In contrast, exposure of the αHβKβMDD mice to hypoxia appeared to increase the intensity of iNOS staining, but this remained in the same region surrounding central veins. Thus, iNOS was expressed in the liver of transgenic mice under ambient room air conditions, but not in normal mice, and it appeared de novo in normal mice made chronically hypoxic, but with a different pattern of distribution in the lobules. These observations suggest that tissue hypoxia is an important stimulant of iNOS expression in the liver.

A number of previous studies have shown that nitric oxide is synthesized in the liver and that under appropriate experimental conditions, hepatocytes express the same inducible isoform of NOS as do macrophages. Furthermore, hepatic iNOS is regulated by factors similar to those that regulate macrophage iNOS, ie, inflammatory cytokines, endotoxin, and glucocorticoids. In a previous immunohistochemistry study of iNOS in rat liver, Buttery et al observed little immunostaining in hepatocytes of rats treated with endotoxin or inflammatory cytokines. The investigators did find strong iNOS immunostaining in scattered macrophages and Kupffer cells. This is in contrast to our observations of iNOS expression in hepatocytes when control mice were exposed to hypoxia. The difference between their observations and ours might be due to the different experimental stimuli used, ie, long-term hypoxia versus acute endotoxin/cytokine exposure or to species differences, ie, rats versus mice. Hypoxia is known to induce iNOS mRNA in rat kidney cells and to increase the gene product and NO release. Hypoxia is also known to upregulate the constitutive isoforms of NOS in endothelial cells and brain. Whether hypoxia leads to iNOS expression in the liver has not been previously reported. Our finding of scattered immunostaining of iNOS in hepatocytes of normal mice exposed to a low oxygen environment suggests that hypoxia also induces liver iNOS.

Histologic studies in isolated hypoperfused normal rat livers have shown differences in the susceptibility of various zones of the hepatic lobule to hypoxic injury. The liver has a high oxygen extraction ratio and an oxygen gradient, with pericentral hepatocytes being exposed to lower oxygen tensions than perportal hepatocytes. Various markers of cell death such as mitochondrial dysfunction, histologic abnormalities, and enzyme release have shown that hepatocytes closest to the central veins are the first to manifest a loss of viability, followed by extension of hypoxic damage towards the perportal areas. In the present study, exposure of normal mice to chronic hypoxia did not lead to any gross or histologic abnormalities in the liver, in contrast to the above-mentioned studies in isolated perfused livers. On the other hand, in the αHβKβMDD mice, hypoxia resulted in multifocal areas of necrosis scattered throughout the liver lobules, sparing with the pericentral zones. This pattern of liver necrosis, including protection of cells around the central vein, is similar to that previously reported in a more severe phenotypic transgenic mouse model,βK and βK-AnNH2. Because the direction of blood flow in the hepatic sinusoids is from the periporal region toward the central veins, PO2 and pH are most likely lowest near the central veins. These conditions would predispose to sickling and adhesion of red blood cells to the endothelium.
HEPATIC NOS IN SICKLE CELL MICE

the sinusoids is expected to reduce oxygen tension even more severely in the region of the central veins. Paradoxically, liver necrosis was not observed in this region in the αβδβ(MD) mice. It has been proposed that NO produced by hepatocytes plays a protective role in the liver by reducing the level of free-oxygen radicals. The balance between NO synthesis and free-oxygen radical formation is thought to determine the degree of hepatocellular toxicity. If NO production is increased in the cells that showed positive iNOS staining, this may account for the absence of necrosis in this region. An alternative explanation is that increased NO production protects hepatocytes by maintaining local blood flow. Weidenbach et al. demonstrated that L-arginine infusion prevents the fall in blood flow in perfused rat livers exposed to endotoxin and PGF2α. Improved blood flow in the region of the central veins might account for protection of the hepatocytes in the αβδβ(MD) mice. Of course, in humans the capacity to induce iNOS expression might be under genetic control, opening the possibility that individual susceptibility involving the NOS system could underlie the interpatient variability in liver damage and propensity to acute hepatic complications.

The colocalization of iNOS immunostaining in the same region showing preserved hepatocytes (Fig IE & F) is consistent with a protective role of iNOS in the pericentral zone. These observations may have significant implications for clinical liver involvement in patients with sickle cell disease and may lead to improved therapy in the future.

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