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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MATERIALS AND METHODS

Animals and tissue preparation. The methods for creating the transgenic sickle cell aHbS(pMDD) mice and their characteristics have been described in previous publications. Briefly, this line of transgenic mice was created by the simultaneous microinjection of

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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 γ-globin gene into
fertilized eggs of the friend virus strain B (FVB) mouse. The
mice with stable coinfection of αα and ββ were then bred onto a
C57BI/6J background for seven or more generations. Higher levels
of human ββ were achieved by breeding the transgenic mice with
C57BI mice bearing a spontaneous deletion of the mouse β major-
globin gene. When the ΔΔ deletion was bred to homozygosity, the
expression of ββ averaged 72.7%. These mice are designated as
ααββ[ββΔΔ] indicating a homozygous deletion of mouse ββΔΔ glo-
bin. Normal mice of the strain C57BI/6J were purchased from Jack-
son 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
paraformaldehydyl-lisine-periodate25 for 10 minutes. Livers were
diseased, 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 sec-
tions 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 immu-
no-staining 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 dewaxed 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 permeabil-
ized 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 antisera to NOS isoforms (Transduction Labs,
Lexington, KY). These antisera were generated against specific pep-
tide sequences of NOS isoforms, and their tissue specificity has been
determined by previous studies in our laboratory.1 In preliminary
experiments, we examined the relative intensity 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 per
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). Optimal color reaction was devel-
oped with diaminobenzidine tetrahydrochloride for 5 minutes.
Sections were then counterstained with hematoxylin, rehydrated with
ethanol, cleared with xylene and coverslipped. For routine histologi-
al analysis, adjacent sections were stained with hematoxylin-eosin
(H&E). Sections from similar lobes of normal and ααββ[ββΔΔ] mice
posed to chronic hypoxia. The sections were coded and analyzed
by two observers blinded to the results with a Nikon Optiphot micro-
scope equipped with a Nikon FX53A camera (Yokohama, Japan).
The following parameters were evaluated: iNOS and EcNOS immu-
nostaining within zones of the hepatic lobule, i.e., 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 ααββ[ββΔΔ] mice housed under room air and processed simulta-
neously under the same conditions.

**Aspartate amino transferase.** Plasma samples were obtained
from C57BI/6J, and ααββ[ββΔΔ] 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 ααββ[ββΔΔ] mice maintained under room air showed normal
histology. In contrast, under chronic hypoxic conditions, the
liver of the ααββ[ββΔΔ] 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 in-
cubated with preimmune serum or absent the primary anti-
sera did not show specific staining. In animals housed under
room air conditions, immunohistochemistry of the liver
showed a diffuse distribution of EcNOS immunostaining
scattered throughout the hepatic lobules of both normal (Fig 1B)
and ααββ[ββΔΔ] mice. There was no change in the pattern
of EcNOS staining in either normal or ααββ[ββΔΔ] mice
when they were exposed to chronic hypoxia. iNOS immuno-
reactivity 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 immunostain-
ing was observed scattered throughout the hepatic lobules
(Fig 1D). In the ααββ[ββΔΔ] transgenic mice, iNOS immuno-
staining 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 in-
tensity of staining appeared to be increased (compare Fig 1E
with 1F).

**Aspartate amino transferase.** In control (C57BI/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 ααββ[ββΔΔ] mice, the level was 61.0 ±
4.3 (n = 20). Although the mean value was higher in the
ααββ[ββΔΔ] mice than in the controls, the difference was
not statistically significant. When two C57BI/6J mice were
exposed to hypoxia for 5 days, there was no apparent change
in plasma amino transferase levels. In contrast, when
ααββ[ββΔΔ] mice were subjected to hypoxia for 5 days, their
plasma level of aspartate amino transferase rose to an aver-
age value of 125.7 ± 9.3 U/mL, respectively (Fig 2). This
value is statistically different from that found for the
ααββ[ββΔΔ] mice under room air (P < .00002). These ele-
ved enzyme levels presumably relate to the abnormal his-
tology found in separate ααββ[ββΔΔ] 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 EeNOS-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 α[ɛ]β[δ][γ[βD1] mouse under room air. (F) Increase in pericentral iNOS in transgenic mouse exposed to hypoxia. Scale bar = 100 μm in A, C, D; 50 μm in B, E, F.
patchy liver necrosis and elevation of plasma aspartate amino transferase (AST) even under ambient condition.

Phenotype, have prominent liver necrosis and elevated aspartate transaminase 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 HbS and HbA mouse. 

Histologic studies in isolated hypoperfused normal rat livers have shown differences in the susceptibility of various zones of the hepatic lobules 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.

To determine whether nitric oxide synthases are expressed in the liver of the sickle cell mice, we performed immunohistochemistry using antibodies directed against endothelial cell 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 

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C57Bl αHββ[βMDD]

Fig 2. Plasma amino transferase levels under ambient (-) and hypoxic (+) conditions for C57Bl and αHββ[βMDD] mice.

**DISCUSSION**

This study has demonstrated that the αHββ[βMDD] mouse model of sickle cell disease develops histologic evidence of 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 HbS and HbA mouse. 

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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 αHββ[βMDD] mice, hypoxia resulted in multifocal areas of necrosis scattered throughout the liver lobules, sparing of 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, HbS and HbA mouse. 

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. Vasoocclusion occurring in...
the sinuoids 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 αββ[IbMMP] 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 PGE2. Improved blood flow in the region of the central veins might account for protection of the hepatocytes in the αββ[IbMMP] 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 1E & 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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