Abnormal modulation of cell protective systems in response to ischemic/reperfusion injury is important in the development of mouse sickle cell hepatopathy

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ABSTRACT

Background
Sickle cell disease, a genetic red cell disorder inherited in an autosomal recessive manner, occurs throughout the world. Hepatic dysfunction and liver damage may be present in sickle cell disease, but the pathogenesis of these conditions is only partially understood.

Design and Methods
Transgenic mice with sickle cell disease (SAD mice) and wild-type mice were exposed to an ischemic/reperfusion stress. The following parameters were evaluated: hematologic profile, transaminase and bilirubin levels, liver histopathology, and mRNA levels of nuclear factor-κB p65, endothelial nitric oxide synthase, inducible nitric oxide synthase, heme oxygenase-1 and phosphodiesterase-1, -2, -3, and -4 genes in hepatocytes obtained by laser-capture microdissection. Immunoblotting was used to analyze the expression of the following proteins: nuclear factor-κB p65 and phospho-nuclear factor-κB p65, heme oxygenase-1, biliverdin reductase, heat shock protein-70, heat shock protein-27 and peroxiredoxin-6. A subgroup of SAD mice was treated with the phosphodiesterase-4 inhibitor rolipram (30 mg/Kg/day by gavage) during the ischemic/reperfusion protocol.

Results
In SAD mice the ischemic/reperfusion stress induced liver damage compatible with sickle cell disease hepatopathy, which was associated with: (i) lack of hypoxia-induced nuclear factor-κB p65 activation; (ii) imbalance in the endothelial/inducible nitric oxide synthase response to ischemic/reperfusion stress; (iii) lack of hypoxia-induced increased expression of heme oxygenase-1/biliverdin reductase paralleled by a compensatory increased expression of heat shock proteins 70 and 27 and peroxiredoxin-6; and (iv) up-regulation of the phosphodiesterase-1, -2, -3, and -4 genes. In SAD mice the phosphodiesterase-4 inhibitor rolipram attenuated the ischemic/reperfusion-related microcirculatory dysfunction, reduced the inflammatory cell infiltration and induced the heme oxygenase-1/ biliverdin reductase cytoprotective systems.

Conclusions
In SAD mice, sickle cell hepatopathy is associated with perturbed nuclear factor-κB p65 signaling with an imbalance of endothelial/inducible nitric oxide synthase levels, lack of heme oxygenase-1/biliverdin reductase expression and up-regulation of two novel cytoprotective systems: heat shock protein-27 and peroxiredoxin-6.

Key words: NF-κB p65, endothelial nitric oxide synthase, inducible nitric oxide synthase, heat shock protein-70, heat shock protein-27, peroxiredoxin-6.

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The online version of this article has a Supplementary Appendix.

Introduction

Sickle cell disease (SCD), an autosomal recessive genetic red cell disorder which occurs throughout the world, results from a point mutation (β^S, 6V) in codon 6 with the insertion of valine in place of glutamic acid leading to the production of a defective form of hemoglobin called hemoglobin S (HbS).[1] Studies of the kinetics of HbS polymerization following deoxygenation have shown it to be a high order exponential function of hemoglobin concentration, thus highlighting the importance of intracellular HbS concentration in sickling.[1,2] Pathophysiological studies have shown that dense, dehydrated red cells play a central role in acute and chronic clinical manifestations of SCD, in which intravascular sickling in capillaries and small vessels leads to vaso-occlusion and impaired blood flow with ischemic cell damage in a variety of organs and tissues.[3,4]

Hepatic dysfunction and liver damage can occur in SCD.[5-11] A review of the literature suggests that ischemic/reperfusion (I/R) injury and a related amplified inflammatory response are of paramount importance in the development of sickle hepatic damage, as in other I/R syndromes (e.g. liver resection, shock and veno-occlusive syndromes).[5,12,13] Studies in these models of I/R liver injury indicate the important role of the homeostasis of liver microcirculation which is under the control of vasoconstrictive molecules such as endothelin-1 (ET-1) and vasodilatory molecules such as nitric oxide (NO) and carbon monoxide.[14] Events that perturb this balanced control result in vasoconstriction of the sinusoidal lumen with reduced local blood flow and tissue oxygenation, further worsened by the entrapment of activated and adherent leukocytes.[14,15]

Pathophysiological studies have shown that the microcirculation is critically involved in the pathogenesis of sickle cell organ damage. In addition, the abnormally activated ET-1 system and the reduced NO bioavaibility associated with activated endothelial vascular cells, highly adherent neutrophils and dense, dehydrated sickle red cells, all participate in sickle cell-related tissue injury.[4,16,17] Although the liver is not one of the main target organs of SCD, its anatomic organization and function, characterized by a sluggish circulation, high metabolic rate and complex regulation of blood flow in the microcirculation, make this an interesting "window organ" to study the pathogenesis of sickle cell-related tissue damage.

Previous studies on sickle cell mouse models exposed to brief periods of acute hypoxia (1-3 h, 7 to 10% oxygen) to mimic acute sickle cell vaso-occlusive crises have shown up-regulation of nuclear factor-κB (NF-κB), increased oxidative stress, reduced local NO bioavailability and modulation of vaso-active molecules.[18-21] Activation of NF-κB has also been shown to be important in other models of I/R liver damage[22-25] and recently, Belcher *et al.* reported functional cross-talk between NF-κB activation and increased expression of heme oxygenase-1 (HO-1), a cytoprotective gene in sickle cell mice.[18]

We, therefore, exposed a transgenic mouse model for SCD, the SAD mice, to an I/R protocol to study the development of sickle cell hepatopathy. Since previous reports have shown beneficial effects of phosphodiesterase (PDE) inhibitors in different model of I/R liver injury (see *Online Supplementary References*) we also studied the effects of a PDE-4 inhibitor, rolipram, on the development of mouse sickle cell hepatopathy.

Design and Methods

Animals

Transgenic Hbb^s/Hbb^s SAD mice (β^{SAD}: β^S, $\beta^{Antilles}$ and $\beta^{D\text{-}Punjab}$) and C57B6/6J control (wild-type, WT) pathogen-free mice were used. The animals (female and male, 20-25 g in body weight) were aged between 4 and 6 months and free from infectious liver diseases.[26] The experiments were carried out in accordance with guidelines from the Italian Ministry of Health and the agreement of the local ethics committee for animal studies.

Ischemic/reperfusion protocol

The I/R injury was induced by hypoxia followed by reoxygenation, as previously reported.[26,27] In brief, WT (n=6) and SAD (n=6) mice were evaluated in ambient air and then after hypoxia (8% O_2) maintained for 4 h (WT and SAD, n=6), 48 h (WT and SAD, n=6) or 168 h (WT and SAD, n=8), followed by 2 h of reoxygenation. No major problems in mouse behavior or significant changes in mouse weight occurred during the I/R protocol. One group of SAD mice (n=6) was treated with the PDE-4 inhibitor rolipram (Sigma-Aldrich Co, St Louis, MO, USA) at the dose of 30 mg/Kg once a day by gavage. Blood sampling and vehicle administration had been previously shown not to affect the blood parameters measured in this study.[26,27] Treatment was started 48 h before hypoxia and maintained during the 168 h of hypoxia. Mice did not show major side effects related to rolipram treatment. In the groups of hypoxic SAD mouse, five of six animals were alive after 48 h of hypoxia and six of eight animals were alive after 168 h of hypoxia, while all WT and rolipram-treated SAD mice were alive and well after 168 h of hypoxia. Mice were given free access to water and food.

Hematologic parameters were measured at baseline and after the different periods of hypoxia, as previously described.[26] Total bilirubin was measured using a Quantichrom Bilirubin Assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions.[28,29] Plasma iron was measured by flame atomic absorption. Blank reagent was processed in parallel and its iron content was subtracted from that of the samples to correct for background iron.[28,30,31] Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) liver enzymes were determined using a spectrometric method (Sigma-Aldrich Co, St Louis, MO, USA).[32]

Histopathology

The liver from sacrificed animals was immediately cut into two parts, one of which was frozen immediately in liquid nitrogen, while the other was fixed in formalin and embedded in paraffin. Multiple (at least five) 3 μm whole mount sections were obtained for each paraffin-embedded liver sample and stained with hematoxylin-eosin, Masson's trichome, and May-Grünwald-Giemsa. A pathological score was determined, as previously described by Duranski *et al.*[32] We also evaluated the inflammatory cell infiltrate and the presence of thrombi. Details on the pathological score are provided in the *Online Supplementary Design and Methods*.

Molecular studies by quantitative reverse-transcription polymerase chain reaction analysis on hepatocytes

In order to study the effects of I/R stress on gene expression in parenchymal cells, we carried out a molecular analysis using a laser capture microdissection (LCM) approach that allowed us to study hepatocytes as a homogenous cell type.[33,34] Hepatocytes identified by cell morphology and isolated by LCM were obtained from frozen liver. LCM was performed on cryostat sections of 8 μm thickness, mounted on 2 μm PEN-membrane coated glass slides (Leica Microsystems, Wetzlar, Germany) and stained with 1:10 diluted hematoxylin (Novocastra, Newcastle upon Tyne,

UK). Two hundred hepatocytes from each liver were cut out and collected in a tube using a DM6000 LCM instrument (Leica) and placed immediately in lysis buffer. We examined the tissue section before and after microdissection to verify the homogeneity of the selected cells.[33,35] Total RNA was isolated from cells obtained by LCM with an RNAqueous kit (Ambion, Foster City, CA, USA), as suggested by the manufacturer. All RNA samples were retrotranscribed to cDNA with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Details on cDNA amplification and quantitative polymerase chain reaction (qPCR) analysis are provided in the *Online Supplementary Design and Methods*. The oligonucleotide primers used in the qPCR are presented in *Online Supplementary Table S1*.

Immunoblot analysis

Twenty cryostat sections of 8 μm thickness obtained from frozen liver samples from mice in each studied groups were lysed with iced lysis buffer (containing 150 mM NaCl, 25 mM bicine, 0.1% SDS, Triton 2%, EDTA 1 mM, protease inhibitor cocktail tablets [Roche], 1 mM Na_3VO_4 final concentration), then centrifuged for 10 min at 4°C at 12,000 g. Proteins were quantified and analyzed by one-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose membranes for immuno-blot analysis with specific antibodies. Details on the antibodies used are provided in the *Online Supplementary Design and Methods*.

Statistical analysis

A two-way ANOVA algorithm for repeated measures between treatment schedules was used for data analysis. Differences with *P* values less than 0.05 were considered statistically significant.

Results

Hypoxia/reoxygenation induced sickle cell-related hepatopathy and was associated with a different pattern of nuclear factor-κB expression in SAD mice

In ambient air SAD mice showed mild liver damage characterized by cytoplasmic vacuolization and focal nuclear pyknosis with some dilated sinusoids and inflammatory cell infiltrate associated with increased liver transaminases (Tables 1 and 2). The main foreseen advantages of using young SAD mice were that these animals: (i) did not have an added thalassemic syndrome, and (ii) had only mild liver damage under normoxic conditions. Thus any changes observed during the I/R stress were not obscured by pre-existing pathology, as observed in old SAD mice or in other mouse models of SCD.

The I/R protocol induced a time-dependent worsening of liver damage in the SAD mice with a significant increase in the pathological score after prolonged hypoxia (Table 1). The histological data were consistent with the development of severe liver damage (after 168 h of hypoxia) recapitulating the elements characterizing sickle cell hepatopathy.[5,10] In the SAD mice, liver transaminases increased progressively with longer periods of hypoxia, reaching a maximum at 168 h of hypoxia, whereas in the WT mice significant changes in AST and ALT levels were observed only after 168 h of hypoxia (Table 2). In SAD mice, the neutrophil count in the peripheral circulation increased significantly after 4 h of hypoxia and red cell density was maximum after 48 h of hypoxia (Table 2), while in WT mice changes in neutrophil count were present only after 48 h of hypoxia, without modifications of red cell density (Table 2). In the late phase of hypoxia, we observed increased hematocrit, hemoglobin levels and reticulocyte count in both mouse strains, which were changes compatible with the effect of hypoxia on erythropoiesis.[27] As we previously reported, prolonged hypoxia also induced a slight worsening of hemolysis in SAD mice, as indicated by the increase in bilirubin and plasma iron levels observed at 168 h of hypoxia only in SAD mice (Table 2).[27]

We then evaluated the effects of I/R injury on NF-κB mRNA levels (*Nfkb*) in laser-captured hepatocytes from both mouse strains. As shown in Figure 1A, in conditions of normoxia, *Nfkb* mRNA levels were significantly lower in SAD hepatocytes than in WT ones. In the early phase of I/R stress (4 h of hypoxia), we observed increased *Nfkb* mRNA levels in hepatocytes from both mouse strains independently of the hematologic phenotype, while in the late phase of I/R, *Nfkb* mRNA levels were down-regulated earlier in SAD hepatocytes than in WT ones, reaching similar values after 168 h of hypoxia in both mouse strains (Figure 1A). We then evaluated the expression of NF-κB p65 and the cellular levels of the active, phosphorylated NF-κB p65 (p-NF-κB p65).[36,37] Under normoxia, we observed higher levels of p-NF-κB p65 levels in SAD mouse livers than in WT mouse livers (Figure 1B). I/R stress induced increased levels of p-NF-κB p65 in WT mice at 4 h of hypoxia reaching a peak at 48 h of hypoxia and becoming undetectable at 168 h of hypoxia (Figure 1B). In

Table 1. Liver pathology of wild-type and SAD mice exposed to hypoxia/reoxygenation and effects of PDE-4 inhibitor (rolipram) treatment.

	WT mice				SAD mice				
	Normoxia	Hypoxia 4 hrs	Hypoxia 48 hrs	Hypoxia 168 hrs	Normoxia	Hypoxia 4 hrs	Hypoxia 48 hrs	Hypoxia 168 hrs	Hypoxia 168 hrs Rolipram
Pathological score	0 (6)	0.5 ± 0.02 (6)	1.0 ± 0.04 (6)*	1.6 ± 0.03(7)*	1.4 ± 0.09 (6)^	1.2 ± 0.05 (6)^	1.7 ± 0.02 (5)^	3.2 ± 0.05 (6)*^	1.8 ± 0.02 (8)°
Inflammatory cell infiltrate	0 (6)	+ (6)	+ (6)	++ (7)	+ (6)	++ (6)	++ (5)	+++ (6)	+ (8)
Thrombi, % (n. of animals)	0% (6)	0% (6)	0% (6)	0% (7)	0% (6)	0% (6)	0% (5)	16% (6)	0% (8)

*Inflammatory cells infiltrate: + : 1-10 cells per field at magnification x 400; ++: 10-50 cells per field at magnification x 400; +++: >50 cells per field at magnification x 400. Pathological score: 0: no hepatocellular damage. 1: mild injury characterized by cytoplasmic vacuolization and focal nuclear pyknosis. 2: moderate injury with dilated sinusoids, cytosolic vacuolization, and blurring of intercellular borders. 3: moderate to severe injury with coagulative necrosis, abundant sinusoidal dilatation, red blood extravasation into hepatic chords, hypereosinophilia and migration of neutrophils. 4: severe necrosis with loss of hepatic architecture, disintegration of hepatic chords, hemorrhage and neutrophil infiltration. Data are presented as means ± SD (n. of experiments); * P< 0.05* versus *normoxia; °P<0.05* versus *168 untreated SAD mice exposed to hypoxia for 168 h; ^P< 0.05* versus *wild-type.*

Table 2. Hematologic and biochemical parameters in wild-type (WT) and SAD mice exposed to hypoxia/reoxygenation and effects of PDE-4 inhibitor (rolipram) treatment.

	WT mice				SAD mice				
	Normoxia (n=6)	Hypoxia 4 hrs (n=6)	Hypoxia 48 hrs (n=6)	Hypoxia 168 hrs (n=7)	Normoxia (n=6)	Hypoxia 4 hrs (n=6)	Hypoxia 48 hrs (n=5)	Hypoxia 168 hrs (n=6)	Hypoxia 168 hrs Rolipram (n=8)
Hematocrit (%)	46.3±0.94	45.8±1.5	44.9±1.2	62.2±2.0*	45.1±1.1	43.7± 0.2	44.8±0.6	52.6±1.8*‡	53.8±1.9 *‡
Hemoglobin (g/dL)	14.8±0.8	15.1±0.4	15.2±1.7	19.2±0.7*	13.8±1.6	12.9±1.5	13.4±0.5	17.4±1.0*‡	16.8±0.86 *‡
Reticulocytes (%)	4.5±1.9	4.2±0.2	5.1±0.8	11.5±3.3*	5.8±0.6	6.1±0.7	5.5±0.3	17.9±2.2*	18.3±1.6*
RBC D_{20}	1.095±0.002	1.093±0.002	1.094±0.001	1.096±0.002	1.102±0.001^	1.106±0.002^	1.108±0.001*^	1.109±0.002*^	1.108±0.002*^
Neutrophils (cell/μL)	851±64.2	1087±124	1853±321*	2658±594*	2188±271^	2569±117	4143±255*	8134±488*‡	4421±877*‡
AST (U/L)	102±11	99.8±14	120±18	197±11°	180±25^	195±11^‡	290±40*‡	450±32*‡	246±22*°‡
ALT (U/L)	98±12	110±8.2	112±25	150±14°	160±21^	193±10^	222±18*	281±41*	189±27°
TBil (mg/dL)	0.2 ±0.05	0.3±0.06	0.2±0.04	0.3±0.08	0.3±0.04	0.35±0.03	0.4±0.03*^	0.5±0.02*^‡	0.4±0.06*
Plasma iron (μg/dL)	147±4.3	-	-	152±7.6	153±5.8	-	-	194±3.9*^	167±10.2

*Data are presented as means ± SD (n of experiments). RBC D_{20}: red cell density expressed as the 20% densest fraction of cells. AST: aspartate aminotransferase: ALT: alanine aminotransferase:TBil: total bilirubin; *P<0.05* versus *baseline; ^P<0.05* versus *wild-type mice under normoxic conditions;°P<0.05* versus *untreated SAD mice exposed to hypoxia for 168 h; ‡P<0.05* versus *wild-type mice exposed to hypoxia for 168 h.*

SAD mice p-NF-κB was undetectable after 4 and 48 h of hypoxia but present at 168 h of hypoxia at a level lower than that observed at baseline but higher than the level in WT mice exposed to the corresponding period of 168 h of hypoxia (Figure 1B).

Since the transcriptional factor NF-κB p65 has been shown to be important in modulation of expression of cytoprotective genes such as endothelial NO synthase (*eNOS*), inducible NO synthase (*iNOS*) and *HO-1* in other models of ischemic liver injury,[38-41] we then evaluated the expression of these genes in livers during the development of sickle cell hepatopathy.

Abnormal modulation of endothelial nitric oxide synthase, heme oxygenase-1 and biliverdin reductase in response to ischemic/reperfusion stress in SAD mouse hepatocytes

Under normoxic condition the SAD mouse hepatocytes showed up-regulation of *eNOS* and *HO-1* compared to WT mice, associated with mild liver damage as a possible response to the chronic oxidative stress related to the sickle cell phenotype (Figure 2). In the early phase of hypoxia *eNOS* mRNA levels were down-regulated in SAD mice, going back to baseline levels after prolonged hypoxia, whereas, in WT mice, *eNOS* mRNA levels were markedly up-regulated in the early phase of I/R stress and then down-regulated to values similar to those observed in SAD mice but still higher than those observed in WT mice under normoxic conditions (Figure 2). *iNOS* mRNA levels were similarly up-regulated in response to I/R stress in both mouse strains (Figure 2). However, hepatocyte *iNOS* expression was down-regulated earlier in SAD mice than in WT mice, indicating a perturbed eNOS/iNOS response to I/R stress in SAD mouse hepatocytes (Figure 2).

We then evaluated *HO-1* mRNA levels in hepatocytes from both mouse strains at the different time points. As reported by Belcher *et al.* under normoxic conditions, *HO-1* mRNA levels were up-regulated in SAD mouse livers compared to WT ones (Figure 2). However, in SAD mice I/R stress induced *HO-1* down-regulation in the early phase of hypoxia followed by up-regulation of *HO-1* after prolonged hypoxia (168 h) (Figure 2). Contrariwise, in WT mice, *HO-1* mRNA levels were significantly increased in

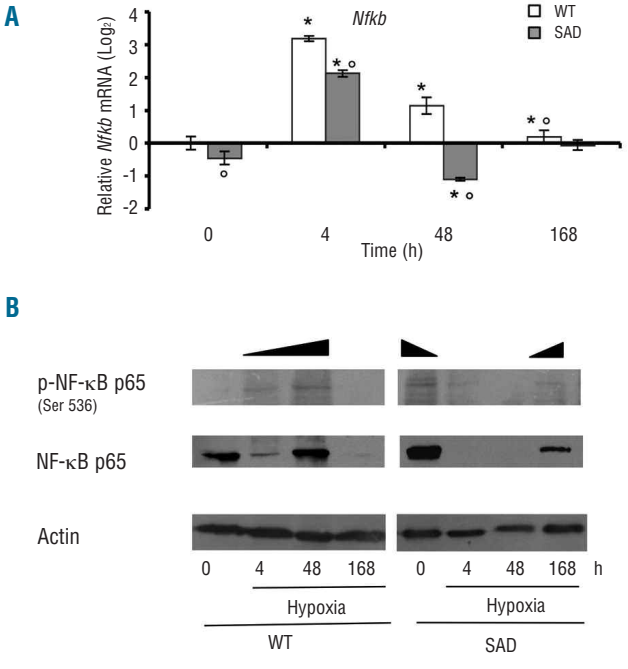

Figure 1. (A) Quantitative RT-PCR expression profile of *Nfkb*, in laser captured hepatocytes from WT mice and SAD mice exposed to I/R stress under normoxia (time 0) and hypoxia (4, 48, and 168 h) followed by 2 h of reoxygenation. Data are presented as means ± SD, n =6-7/group; * *P*<0.05 compared to baseline values; ° *P*<0.05 compared to WT mice; ^ *P*< 0.05 compared to untreated SAD mice. The gene expression levels recorded after different experimental conditions were normalized using the average of the expressions of *Gapdh* and *rRNA18s* as an endogenous reference. Data were calculated by the comparative method. (B) Immunoblot analysis with specific anti-phospho-NF-κB p65 and anti-NF-κB p65 antibody of hepatocytes from WT and SAD mice under normoxia (time 0) and hypoxia (4, 48, and 168 h) followed by 2 h of reoxygenation. A representative experiment of six performed with similar results is shown.

the early phase of I/R stress and down-regulated in the late phase of I/R damage (Figure 2).

HO-1 protein expression in response to I/R stress was significantly higher in SAD mouse liver than in WT mouse liver (Figure 3). In the early phase of I/R stress we observed a significant reduction in HO-1 protein expression in SAD mice compared to that in either SAD mice at baseline or control mice after the same period of hypoxia (Figure 3). At 48 h of hypoxia, HO-1 protein expression in SAD mice was increased to values similar to those observed at baseline, which were maintained at 168 h of hypoxia in the presence of sickle cell hepatopathy. In WT mice HO-1 protein expression was reduced after 4 h of hypoxia and then increased, such that its levels were higher at 48 and 168 h than either baseline levels or levels in SAD mice (Figure 3).

In the light of previous reports showing a functional link between HO-1 and biliverdin reductase (BVR) (see *Online Supplementary References*), we evaluated BVR protein expression in SAD mouse livers under steady state and during I/R stress. In normoxic conditions, as observed for HO-1, BVR protein expression was significantly increased in SAD mice compared to in WT animals (Figure 3). During I/R stress, BVR expression was reduced early (at 4 h) of hypoxia in both mouse strains but then markedly increased in WT mice but not in SAD mice in which the I/R liver damage was more severe.

We then examined whether the perturbations in HO-1 and BVR responses to I/R also occurred in other cytoprotective systems such as the heat shock proteins (HSP), which have been shown to cross-talk with NF-κB, to parallel HO-1 expression and to increase in response to I/R stress in other models of ischemic liver damage (see *Online Supplementary References*). We evaluated the expression of HSP70 and HSP27 in mouse livers from both mouse strains exposed to I/R stress. We observed similar expression of HSP70 in SAD and control mouse livers under normoxia (Figure 3). I/R induced increased HSP70 expression in the early phase of hypoxia (at 4 h) in both mouse strains, and the levels of expression were similarly reduced after 48 h of hypoxia (Figure 3). In SAD mice, the prolonged I/R stress induced a further increase in HSP70 expression, while WT mice showed a marked reduction of HSP70 levels at 168 h of hypoxia compared to the levels of either normoxic WT mice or SAD mice exposed to hypoxia for 168 h (Figure 3). HSP27 expression was reduced at 4 and 168 h of hypoxia in WT mice, while no significant differences were observed in HSP27 expression in SAD mice exposed to I/R stress. However, the relative increased expression of HSP27 in SAD mice after 168 h of hypoxia was significantly different to that in the group of WT mice exposed to hypoxia for the same period (Figure 3).

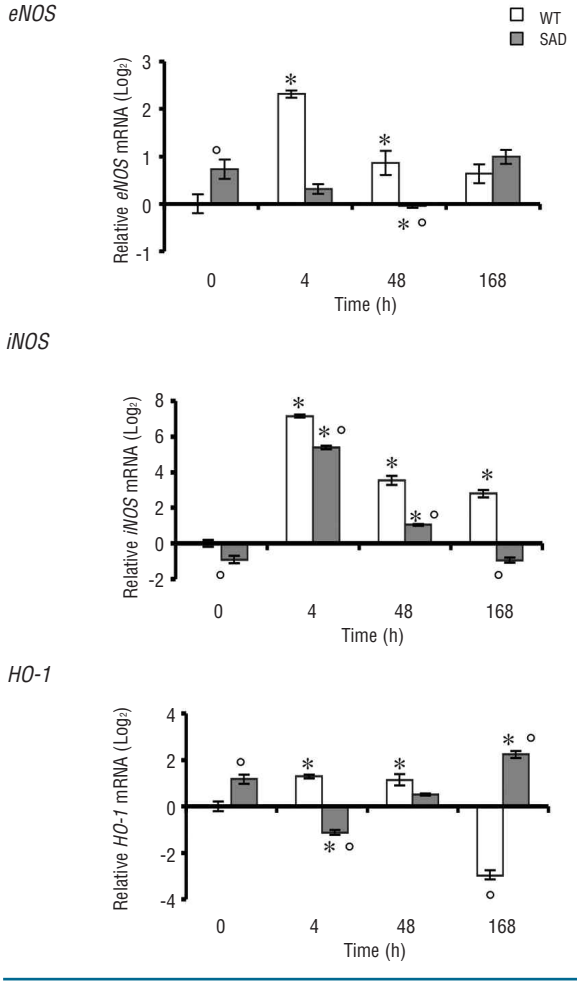

Figure 2. Quantitative RT-PCR expression profile of *eNOS* (endothelial NO synthase), *iNOS* (inducible NO synthase), and *HO-1* (heme oxygenase-1), in laser-captured hepatocytes from WT mice and SAD mice under normoxia (time 0) and hypoxia (4, 48, and 168 h) followed by 2 h of reoxygenation. Data are presented as means±SD, n =6-7 mice/group; * $P<0.05$ compared to baseline values; ° $P<0.05$ compared to WT mice. For each gene, the expression levels found after different experimental conditions were normalized using the average of the expressions of *Gapdh* and *rRNA18s* as an endogenous reference. Data were calculated by the comparative method.

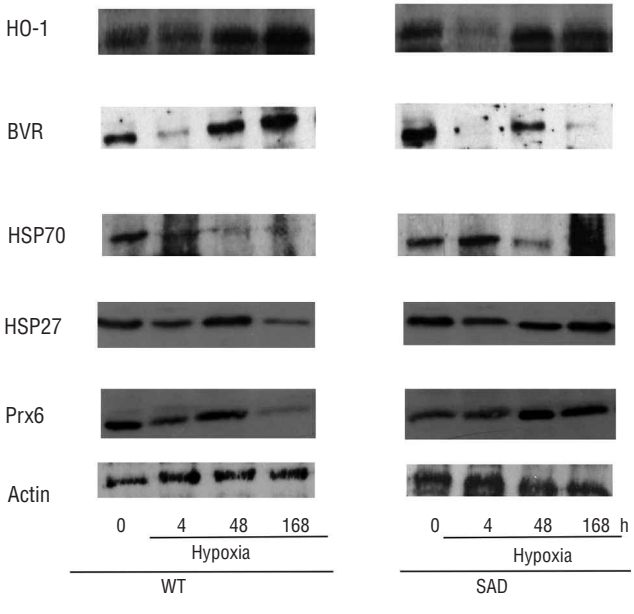

Figure 3. Immunoblot analysis of heme oxygenase-1 (HO-1), biliverdin reductase (BVR), heat shock protein 70 (HSP70), heat shock protein 27 (HSP27) and peroxiredoxin-6 (Prx6) expression in livers from WT and SAD mice under normoxia (time 0) and hypoxia (4, 48, and 168 h) followed by 2 h of reoxygenation. A representative experiment of six performed with similar results is shown. Expression of actin was used as a protein-loading control.

Previous *in vitro* and *in vivo* studies demonstrated the hepatoprotective effects of Prx6 in different models of liver injury caused by oxidative insults or I/R stress (see *Online Supplementary References*). In this study we showed that Prx6 expression in normoxic conditions was lower in SAD mice than in WT ones (Figure 3). After 4 h of hypoxia, Prx6 expression was significantly decreased in WT mice, while no changes occurred in the SAD mouse group (Figure 3). After 48 h of hypoxia, Prx6 expression in WT mice increased, reaching values similar to those observed at baseline, while Prx6 expression in SAD mice was significantly increased compared to that in either normoxic SAD mice or WT mice after 48 h of hypoxia. In the SAD mice, prolonged hypoxia (168 h) caused a significant increase in Prx6 expression compared to the level present in normoxic animals, while in the WT mice, the prolonged hypoxia markedly reduced Prx6 expression compared to both baseline values and levels after other shorter periods of hypoxia (Figure 3).

Since previous studies in other models of hepatic I/R injury showed possible beneficial effects of PDE inhibitors[42] (see *Online Supplementary References*) we first evaluated the expression of *PDE* genes in both mouse strains under normoxia and I/R stress and then administered the PDE-4 inhibitor rolipram to compared the effects of treatment on hepatopathy induced by prolonged hypoxia in SAD mice.

Ischemia/reperfusion induced up-regulation of the expression of genes for phosphodiesterase-1, -2, -3 and -4 isoforms

Since different PDE isoforms are involved in hydrolyzation of cyclic nucleotides with overlapping effects, we evaluated the expression of the PDE-1, -2, and -3 families which hydrolyze both cAMP and cGMP as substrates but with different affinities, and the PDE-4 family that hydrolyzes cAMP.[27] cAMP and cGMP, acting as second messengers in response to extracellular stimuli, are important in the regulation of vascular tone and in the modulation of neutrophil chemotaxis.[27] The following isoforms were undetectable in hepatocytes from both mouse strains: *PDE3a* (111839), *PDE4a* (115458-69577-39413), *PDE4b* (106911), *PDE4c* (34307-110095) and *PDE4d* (74103-79975). Some differences in PDE response to hypoxia were observed between WT and SAD mice (Figure 4). Of interest all PDE-4 isoforms were up-regulated in early hypoxia and returned to baseline values in both WT and SAD mice after prolonged hypoxia (Figure 4C).

Based on these observations, we administrated an inhibitor of PDE-4 isoforms (rolipram) to SAD mice that developed sickle cell hepatopathy, corresponding to SAD mice exposed to hypoxia for 168 h.

Rolipram has beneficial effects on sickle cell hepatopathy and modulates cytoprotective systems

SAD mice treated with the PDE-4 inhibitor rolipram and then exposed to I/R stress showed a significant reduction of liver pathological score, serum liver transaminases and liver inflammatory cell infiltrates compared to either untreated hypoxic SAD mice or hypoxic WT mice (Table 1, Figure 5A). We also observed a significant reduction in total neutrophil counts in these mice compared to untreated hypoxic SAD mice, suggesting a systemic anti-inflammatory effect of the PDE-4 inhibitor (Table 2).

The improvement of sickle cell-related liver damage in SAD mice treated with rolipram compared to the damage in untreated hypoxic SAD mice was also associated with: (i) reduced NF-κB p65 activation; (ii) increased *eNOS* mRNA levels, and (iii) increased HO-1 and BVR protein expression associated with marked reduction in HSP70 expression and no changes in HSP27 and Prx6 proteins levels (Figure 5B-D).

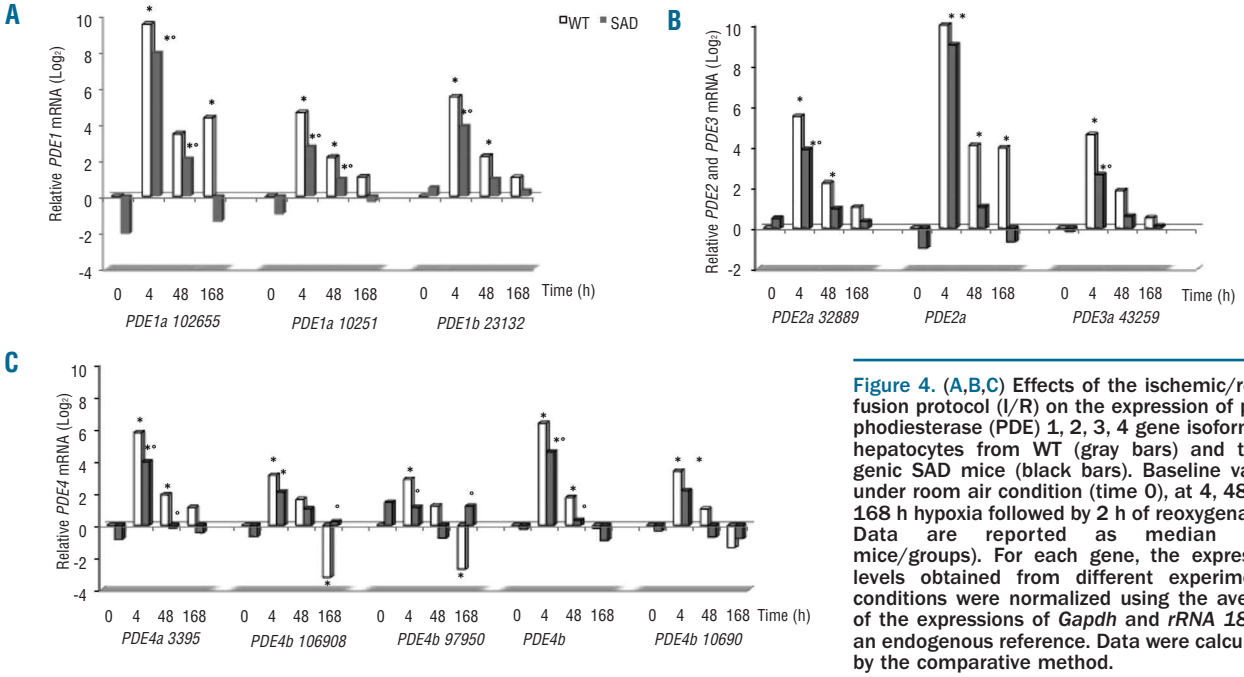

Figure 4. (A,B,C) Effects of the ischemic/reperfusion protocol (I/R) on the expression of phosphodiesterase (PDE) 1, 2, 3, 4 gene isoforms in hepatocytes from WT (gray bars) and transgenic SAD mice (black bars). Baseline values under room air condition (time 0), at 4, 48 and 168 h hypoxia followed by 2 h of reoxygenation. Data are reported as median (n=6 mice/groups). For each gene, the expression levels obtained from different experimental conditions were normalized using the average of the expressions of *Gapdh* and *rRNA 18S* as an endogenous reference. Data were calculated by the comparative method.

Discussion

Here we show that normoxic SAD mice have mild, chronic hepatopathy associated with increased cellular levels of p-NF-κB p65 in hepatocytes and up-regulation of cytoprotective genes such as *eNOS* and *HO-1* together with increased HO-1/BVR protein expression possibly induced to limit the liver damage but most likely insufficient to fully counterattack the chronic damage related to SCD.[14,43,44] Other protective stress-response systems, such as the molecular chaperones HSP70 and HSP27 and the endogenous anti-oxidant Prx6 (see *Online Supplementary References*), were similarly expressed in both mouse strains in conditions of normoxia, suggesting that these two HSP and Prx6 are not involved in protecting murine liver cells against chronic sickle cell-related damage.

In SAD mice exposed to I/R stress to induce sickle cell hepatopathy we studied the molecular mechanisms involved in the liver damage of SCD. The development of severe sickle cell hepatopathy was characterized by temporal differences in NF-κB p65 activation in SAD mice compared to in WT mice in response to I/R stress. Previous studies in other models of liver injury showed the important role of NF-κB p65 in signal transduction towards cytoprotective genes such as *eNOS*, *iNOS* and *HO-1*.[38,45,46] In SAD mice, we observed a lack in hypoxia-induced eNOS expression in the early phase of I/R stress compared to that in WT mice while the pattern of iNOS expression in response to I/R stress was similar in both mouse strains. Previous studies have shown the importance of controlled balance between iNOS/eNOS levels in local NO homeostasis and microvascular tone regulation to reduce the I/R liver injury.[47,48] In SAD mice the imbalance between iNOS/eNOS expression in response to I/R stress may unfavorably affect NO bioavailability, contributing to the dysfunctional liver microcirculation involved in the development of sickle hepatopathy (Figure 3). In addition, the response of other cytoprotective systems, such as HO-1, BVR, HSP and Prx6, to I/R stress also differs between SAD mice and WT ones, representing a new additional factor possibly increasing the susceptibility of the liver of SAD mice to I/R stress. The lack of HO-1 and BVR up-regulation in response to I/R stress observed in SAD mice might contribute to the development of more severe cellular damage beside the biphasic increase of HSP70, which seems not to be sufficient to counteract the sickle cell-related acute organ injury (Figure 5). In SAD mice, expression of both HSP27 and Prx6 was increased after prolonged hypoxia when compared with that in WT mice, which showed significantly reduced expression of both proteins. Although HSP27 and Prx6 are members of different functional groups (molecular chaperones and anti-oxidant systems, respectively), they are both induced in response to oxidative stress in other cell types and their low expression increases cell susceptibility to oxidative stress[49] (see also *Online Supplementary References*). Our data, supported by previously published evidence, indicate a possible novel hepatoprotective role of HSP27 and Prx6 in SAD mice exposed to prolonged I/R stress.

Previous studies in various models of I/R-induced liver injury have shown beneficial effects of PDE inhibitors[50] (see *Online Supplementary References*). In this study we showed that PDE-1, -2, -3, and -4 were up-regulated in the early phase of hypoxia (4 h) in both mouse strains, indicating that these PDE isoforms are involved in the cellular response to I/R cell injury. Blocking the PDE-4

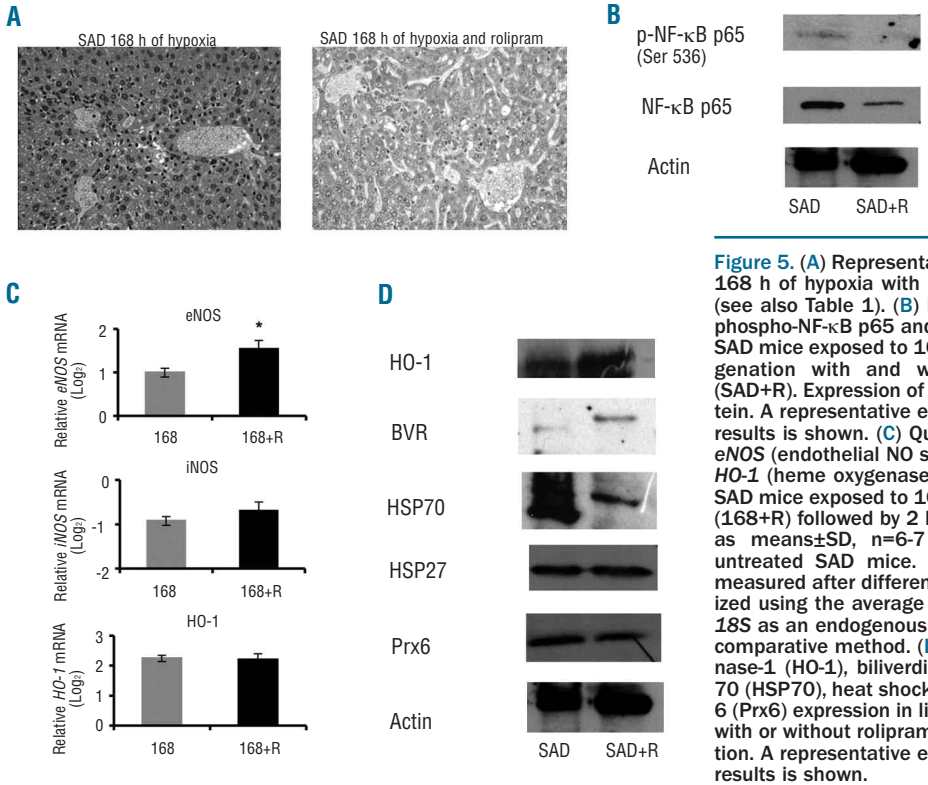

Figure 5. (A) Representative examples of SAD mouse liver after 168 h of hypoxia with or without the PDE-4 inhibitor rolipram (see also Table 1). (B) Immunoblot analysis with specific anti-phospho-NF-κB p65 and anti-NF-κB p65 antibody of livers from SAD mice exposed to 168 h of hypoxia followed by 2 h of reoxygenation with and without the PDE-4 inhibitor rolipram (SAD+R). Expression of actin was used as a loading control protein. A representative experiment of six performed with similar results is shown. (C) Quantitative RT-PCR expression profile of *eNOS* (endothelial NO synthase), *iNOS* (inducible NO synthase), *HO-1* (heme oxygenase-1), in laser-captured hepatocytes from SAD mice exposed to 168 h of hypoxia with or without rolipram (168+R) followed by 2 h of reoxygenation. Data are presented as means±SD, n=6-7 mice/group; **P*<0.05 compared to untreated SAD mice. For each gene, the expression levels measured after different experimental conditions were normalized using the average of the expressions of *Gapdh* and *rRNA 18S* as an endogenous reference. Data were calculated by the comparative method. (D) Immunoblot analysis of heme oxygenase-1 (HO-1), biliverdin reductase (BVR), heat shock protein 70 (HSP70), heat shock protein 27 (HSP27) and peroxiredoxin-6 (Prx6) expression in livers from SAD mice exposed to hypoxia with or without rolipram (SAD+R) followed by 2 h of reoxygenation. A representative experiment of six performed with similar results is shown.

family of proteins by rolipram allowed SAD mice to survive prolonged hypoxia and improved the sickle cell-related liver injury as indicted by: (i) the low liver pathological score; (ii) the marked decrease in local and systemic cell inflammatory response; (iii) the up-regulation of *eNOS* gene expression balancing *iNOS* expression; and (iv) the increased HO-1/BVR expression with reduced HSP70 levels. These data suggest that in SAD mice the inhibition of PDE-4 attenuates the microcirculatory dysfunction related to I/R stress, reduces the inflammatory cell infiltration and activates the HO-1/BVR cytoprotective systems, which in turn reduces NF-κB p65 activation with improvement of hepatocellular survival. Further studies should be carried out to better elucidate the functional networks activated in response to I/R stress and involved in the pathogenesis of organ damage in SCD since these may be possible targets for the development of new therapeutic strategies.

Authorship and Disclosures

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