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

Angela Siciliano¹, Giorgio Malpeli², Orah S. Platt³, Christophe Lebouef⁴,⁵,⁶, Anne Janin⁴,⁵,⁶, Aldo Scarpa², Oliviero Olivieri¹, Eliana Amato², Roberto Corrocher¹, Yves Beuzard⁷, and Lucia De Franceschi¹

¹Department of Medicine, University of Verona; ²Department of Pathology, University of Verona, Verona, Italy; ³Lab of Medicine and Pathology, Children’s Hospital, Harvard Medical School, Boston, MA, USA; ⁴Inserm, U728, Paris, F-75010, France; ⁵Université Paris 7- Denis Diderot, Paris, France; ⁶AP-HP, Hôpital Saint-Louis, F-75010, Paris, France, and ⁷Laboratory of Gene Therapy and Hematological diseases, INSERM U-733, Hospital Saint Louis, Paris, France

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

Running title: hepatoprotective systems in SCD

Correspondence
Lucia De Franceschi, MD, Dept. of Medicine, Section of Internal Medicine
University of Verona, Policlinico GB Rossi; P.le L Scuro, 10; 37134 Verona, Italy.
Phone: international +39.045.8074918. Fax: international +39 045.8027473.
E-mail: lucia.defranceschi@univr.it
Background and Objectives. Sickle cell disease is a worldwide-distributed autosomal recessive genetic red cell disorder. Hepatic dysfunction and liver damage is present in sickle cell disease, but its pathogenesis is only partially known.

Design and Methods. Transgenic mouse model for sickle cell disease (SAD mice) and wild-type mice were exposed to ischemic/reperfusion protocol. The following parameters were evaluated: hematological profile, transaminases and bilirubin levels, liver pathology, mRNA levels of nuclear factor-kB p65, endothelial NO synthase, inducible NO synthase, heme oxygenase-1 and phosphodiesterase-1-2-3-4 genes in hepatocytes obtained by laser-capture-microdissection. Protein expression of nuclear factor-kB p65 and phospho-nuclear factor-kB p65, heme oxygenase-1, biliverdin reductase, heat-shock-protein-70,-27 and peroxiredoxin-6 were analysed by immunoblot. A subgroup of SAD mice was treated with the phosphodiesterase-4 inhibitor Rolipram (30 mg/Kg/day by gavage) during the ischemic/reperfusion stress.

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-kB p65 activation; (ii) unbalance in endothelial /inducible NO synthase response to ischemic/reperfusion stress; (iii) lack of hypoxia induced heme oxygenase-1/ biliverdin reductase increased expression paralleled by the compensatory increased in heat-shock-protein-70,-27 and peroxiredoxin-6 expression; (iv) up-regulation of the phosphodiesterase-1,-2,-3,-4 genes. In SAD mice the phosphodiesterase-4 inhibitor Rolipram attenuates the ischemic/reperfusion related microcirculatory dysfunction, reduces the inflammatory cell infiltration and induced the heme oxygenase-1/ biliverdin reductase cytoprotective systems.

INTRODUCTION

Sickle cell disease (SCD) is a worldwide-distributed autosomal recessive genetic red cell disorder, which 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, the hemoglobin S (HbS) (1-3). Studies of the kinetics of HbS polymerization following deoxygenation have shown it to be a high order exponential function of hemoglobin concentration, thus highlighting a crucial role for cellular HbS concentration in sickling (4,5). Pathophysiological studies have shown that the 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 (4-7).

Hepatic dysfunction and liver damage is present in SCD (8-14). A revision of the literature suggest the crucial role of ischemic/reperfusion (I/R) injury and related amplified inflammatory response in the development of sickle hepatic damage similarly to what observed in other I/R syndromes (i.e.: liver resection, shocks or veno-occlusive syndromes) (8, 15, 16). Studies in these models of I/R liver injury indicate the important role of liver microcirculation that is placed under the controlled balance between vasoconstritive molecules as endothelin-1 (ET-1) and vasodilatative molecules as nitric oxide (NO) and carbon monoxide (CO) (17). Events that perturbate this balance 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 (17,18).

Pathophysiological studies have shown that microcirculation is critically involved in the pathogenesis of sickle cell organ damage. In addition, the abnormally activated ET-1 system and the reduced NO bioavailability associated with activated endothelial vascular cells, highly adherent neutrophils and dense, dehydrated sickle red cells, participate all in sickle cell related tissue injury (6,19-21). Although 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 an elaborated microcirculation blood flow regulation allowed us to consider liver as an interesting “window organ” to study the pathogenesis of sickle cell related tissue damage.

Previous studies on sickle cell mouse models exposed to short time acute hypoxia (1-3 hours 7 to 10% oxygen) to mimic acute sickle cell vaso-occlusive crisis (VOCs) have shown up-regulation of nuclear factor-kB (NF-kB), increased oxidative stress, reduced local NO bioavailability and modulation of vaso-active molecules (22-25). The activation of NF-kB has also been shown to be important in other models of I/R liver damage (26-29) and recently, Belcher et al have reported functional cross-talk between NF-kB activation and increased expression of heme oxygenase-1 (HO-1) as cytoprotective gene in sickle cell mice (22).

Here, we exposed a transgenic mouse model for SCD, the SAD mice, to I/R protocol and we observed the development of sickle cell hepatopathy, which was associated with perturbation of NF-kB p65 activation, imbalance of I/R induced endothelial NO synthase/inducible NO synthase expression, lack of HO-1/biliverdin reductase (BVR) response to I/R stress and modulation of chaperone and anti-oxidant systems. Since previous studies have shown beneficial effects of phosphodiesterase (PDE) inhibitors in different model of I/R liver injury (30-34), we treated SAD mice with the PDE-4 inhibitor, Rolipram, that prevented the development of sickle hepatopathy most likely through the modulation of microvascular tone and inflammatory cell response associated with increased expression of cytoprotective systems.
DESIGN AND METHODS

Animals
Transgenic Hbb\textsuperscript{S}/Hbb\textsuperscript{S} SAD mice (\(\beta\text{SAD, } \beta\text{S Antilles and } \beta\text{D-Punjab}\)) and C57B6/6J as control (wild-type, WT) pathogen-free mice aged between 4 and 6 months and free from liver infectious disease were used (female and male, 20-25 g in body weight) (35). The experiments were carried out in accordance with guidelines from the Italian Ministry of Health and the agreement of the local ethic committee for animal studies.

Ischemic/reperfusion (I/R) protocol
Hypoxia followed by 2 hours (hrs) of reoxygenation was used to induce ischemic/reperfusion injury (I/R) as previously reported (35, 36). WT (\(n=6\)) and SAD (\(n=6\)) mice were evaluated under ambient air condition and at 4 (WT and SAD \(n=6\)), 48 (WT and SAD \(n=6\)) and 168 (WT and SAD \(n=8\)) hours (hrs) hypoxia (8% O\(_2\)) followed by 2 hrs reoxygenation. No major problems in mouse behaviour or significant changes in mouse weight were present during the I/R protocol. One group from SAD mouse strain (\(n=6\)) was treated with the PDE-4 inhibitor Rolipram (Sigma-Aldrich Co, St Louis, MO; USA) at the dosage of 30 mg/Kg once a day by gavage. Blood sampling and vehicle administration have been previously shown not to affect the blood parameters measured in this study (35, 36). Treatment started 48 hrs before hypoxia and maintained during 168 hrs hypoxia. Mice did not show major side effects related to Rolipram treatment. In hypoxic SAD mouse groups, 5 of 6 animals were alive at 48 hours hypoxia and 6 of 8 animals were alive at 168 hours hypoxia, while all WT and Rolipram treated SAD mice were alive and well after 168 hrs hypoxia. Mice were given free access to water and food.

Hematological parameters were measured at baseline and at the different time point hypoxia as previously described (35). Total bilirubin was measured using Quantichrom Bilirubin Assay kit (BioAssay Systems, Hayward, Ca; USA) according to the manufacturer instructions (37, 38). 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 (37, 39, 40). Liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using spectrometric method (Sigma-Aldrich Co, St Louis, MO; USA) (41).

**Histopathology**

*Liver tissue histology:* Liver was immediately cut into two parts, one immediately frozen in liquid nitrogen, while the other was fixed in formalin and embedded in paraffin. Multiple (at least five) three microns whole mount sections were obtained for each paraffin-embedded liver and stained with hematoxylin eosin, Masson’s trichome, and May-Grünwald-Giemsa. 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, haemorrhage and neutrophils infiltration (41, 42). We also evaluated the inflammatory cell infiltrate and the presence of thrombi. Morphologic analysis was performed blindly and independently by two pathologists and consisted of the evaluation of the tissue architecture and changes induced by hypoxia and/or treatment regimens. The inter-observer difference measure was <5%.
Molecular studies by quantitative RT (reverse-transcription)-PCR (qPCR) analysis on laser capture microdissection (LCM) hepatocytes

In order to study the effects of I/R stress on gene expression in parenchimal cells, we carried out the molecular analysis using the laser capture microdissection approach that allowed us to study hepatocytes as homogenous cell type (43,44). Hepatocytes identified by cell morphology and isolated by laser capture microdissection (LCM) were obtained from frozen liver. LCM was performed on cryostatic sections of 8 \( \mu \)m of thickness, mounted on 2 \( \mu \)m PEN-membrane coated glass slides (Leica Microsystems, Wetzlar, Germany) and stained with 1:10 diluted hematoxylin (Novocastra, Newcastle upon Tyne, UK). Two hundred hepatocytes for each liver were cut out and collected in a tube by a DM6000 LCM instrument (Leica) and placed immediately in lyses buffer. We examined the tissue section before and after microdissection to verify the homogeneity of the selected cells (43, 45). Total RNA was isolated from cells obtained by LCM with RNAqueous kit (Ambion, Foster City, CA, USA), as suggested by manufacturers. All RNAs samples were retrotranscribed to cDNA with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). cDNAs of cells isolated by LCM were pre-amplified by 14 PCR cycles (each cycle of 15 sec at 95°C and 240 sec at 60°C) in a solution including 0.05X Taqman probes or 50 nM forward and reverse oligonucleotide primers, 5 \( \mu \)L of cDNA and 1x Taqman PreAmp Master Mix (Applied Biosystems). The pre-amplified solution was diluted 1/20 and 5 \( \mu \)L of the dilution were used as a template for subsequent qPCR, in the presence of 1/20 Taqman probe and 1x Taqman Universal Master Mix (Applied Biosystems) or 1x Power SYBR Green Master Mix and 400 nM each primer. All qPCR reactions were performed in 20 \( \mu \)L final volume. Thermal cycling included in all cases an initial incubation at 95 °C for 10 minutes than 40 PCR cycles (15 sec at 95 °C and 60 sec at 60°C). Samples were analyzed in triplicate on ABI Prism 7900 SDS instrument (Applied Biosystems). Oligonucleotide primers used in qPCR are shown in Supplemental Table 1S. The relative gene expression level was calculated by the comparative method using the average of the expressions of
Gapdh (Taqman probe Mm99999915_g1; Applied Biosystems) and rRNA 18S (Hs99999901_s1) as an endogenous reference. Data were analyzed as indicated in User Bulletin #2 (Applied Biosystems).

**Immunoblot-analysis**

Twenty cryostatic sections of 8 µm of thickness obtained from each mouse frozen livers from each studied groups were lysed with iced lyses buffer (LB containing: 150 mM NaCl, 25 mM bicine, 0.1% SDS, Triton 2%, EDTA 1 mM, protease inhibitor cocktail tablets (Roche), 1 mM Na₃VO₄ final concentration) then centrifuged 10 min at 4°C at 12,000 g. Proteins were quantified and analysed by mono-dimensional SDS polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose membranes for immuno-blots analysis with specific antibody against Nuclear Factor-kB p65 (NF-kB p65, clone C22B4, Cell Signaling), phospho-Nuclear Factor-kB p65 (p-NF-kB p65, Ser 536, Cell Signaling), heme-oxygenase-1 (HO-1; SC-10789; Santa Cruz Biotechnology, Santa Cruz, CA, USA), biliverdin reductase (BVR; Assay Designs), Heat Shock Protein 70 (HSP70, clone K-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Heat Shock Protein 27 (HSP27; SC-1048; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Peroxiredoxin-6 (Prx6; Sigma Chemical Co, St Louis, MO; USA) and anti-actin (Sigma Chemical Co, St Louis, MO; USA). Actin was used as loading control.

**Statistical analysis**

The 2-way ANOVA algorithm for repeated measures between treatment schedules was used for data analysis. Differences with p<0.05 were considered significant.
RESULTS

Hypoxia/reoxygenation (I/R) induced sickle cell related hepatopathy and is associated with different NF-kB expression pattern in SAD mice

Under ambient air condition 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 (Table 1, 2). The main foreseen advantages of the use of SAD mouse strain were: (i) the animals do not have an added thalassemic syndrome, and (ii) the SAD mice showed a mild liver damage under normoxic condition, thus any changes observed during the I/R stress are not obscured by previous ones as observed in aged SAD mice or in other mouse model for SCD.

The I/R protocol induced a time dependent worsening of liver damage in SAD mice with significant increased pathological score after prolonged hypoxia (Table 1). The histological data were consistent with the development of a severe liver damage (at 168 hrs hypoxia) recapitulating the elements characterizing the sickle cell hepatopathy (8, 13). At the different time points of hypoxia, in SAD mice liver transaminases progressively increased, reaching a peak at 168 hrs hypoxia; whereas, in WT mice we observed significant changes in AST and ALT levels only after 168 hrs hypoxia (Table 2). In SAD mice, we observed a significant increased in neutrophil count in peripheral circulation after 4 hrs hypoxia and in the densest red cells after 48 hours hypoxia (Table 2), while in WT mice changes in neutrophil count were present only after 48 hrs hypoxia, without modifications of red cell density (Table 2). In the late phase hypoxia, we observed in both mouse strains increased hematocrit, hemoglobin levels and reticulocyte count, which were compatible with the effect of hypoxia on erythropoiesis (36). As we previously reported prolonged hypoxia induced also a slight worsening of hemolysis in SAD mice as supported by the increased in bilirubin and plasma iron levels observed at 168 hrs hypoxia only in SAD mice (Table 2) (36).
We then evaluated the effects of I/R injury on NF-kB (Nfkβ) mRNA levels in laser-captured hepatocytes from both mouse strains. As shown in Fig. 1a, in normoxic SAD hepatocytes, Nfkβ mRNA levels were significantly lower compared to WT ones (Fig. 1a). In the early phase of I/R stress (4 hrs hypoxia), we observed increased Nfkβ mRNA levels in hepatocytes from both mouse strains independently from the hematological phenotype, while in the late phase of I/R Nfkβ mRNA levels were earlier down-regulated in SAD hepatocytes than in WT ones, reaching similar values after 168 hrs hypoxia in both mouse strains (Fig. 1a). We then evaluated the expression of NF-kB p65 and the cellular levels of phosphorylated (active) NF-kB p65 (46, 47). Under normoxia, we observed increased p-NF-kB p65 levels in SAD mouse livers compared to WTs (Fig. 1b). I/R stress induced increased levels of active p-NF-kB p65 in WT mice at 4 hrs hypoxia reaching a peak at 48 hrs hypoxia and becoming undetectable at 168 hrs hypoxia (Fig. 1b). In SAD mice p-NF-kB was undetectable at 4 and 48 hrs hypoxia but present at 168 hrs hypoxia to levels lower than those observed at the baseline but higher than the corresponding 168 hrs hypoxic WT mouse group (Fig. 1b).

Since the transcriptional factor NF-kB p65 has been shown to be important in modulation of cytoprotective gene expression such as eNOS, iNOS and heme-oxygenase-1 in other model of ischemic liver injury (48-51), we then evaluated the expression of these genes in livers during the development of sickle cell hepatopathy.

**Abnormal modulation of eNOS, heme oxygenase-1 and biliverdin reductase in response to I/R stress in SAD mouse hepatocytes**

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

We then evaluated the heme-oxygenase 1 (*HO-1*) mRNA levels in hepatocytes from both mouse strains at the different time points. As reported by Belcher et al under normoxic condition, we observed an up-regulation of *HO-1* mRNA levels in SAD mouse livers compared to WTs (Fig. 2). However, in SAD mice I/R stress induced a *HO-1* down-regulation in the early phase of hypoxia followed by up-regulation of *HO-1* gene expression after prolonged hypoxia (168 hrs) (Fig. 2). Otherwise, in WT mice, *HO-1* mRNA levels were significantly increased in the early phase of I/R stress and down-regulated in the late phase of I/R damage (Fig. 2).

We then evaluated HO-1 protein expression in response to I/R stress in liver from both mouse strains. As shown in Fig. 3, under normoxic condition HO-1 protein expression was significantly higher in SAD mice compared to WTs. In the early phase of I/R stress we observed a significant reduction in HO-1 protein expression in SAD mice compared to either SAD mice at baseline or control mice at the same time interval (Fig. 3). At 48 hrs hypoxia, in SAD mice HO-1 protein expression was increased to values similar to those observed at baseline, which were maintained at 168 hrs hypoxia in presence of sickle cell hepatopathy. In WT mice HO-1 protein expression was reduced at 4 hours hypoxia followed by increased expression at 48 and 168 hrs compared to either baseline levels or SAD mice (Fig.3).

Based on previous reports showing functional link between HO-1 and biliverdin reductase (BVR) (50-53), we evaluated BVR protein expression in SAD mouse livers under steady state and during I/R stress. Under normoxia, BVR protein expression was significantly increased in SAD mice compared to WTs similarly to
what observed for HO-1 (Table 2, Fig. 3). During I/R stress, BVR expression was early reduced at 4 hrs hypoxia in both mouse strains but then markedly increased only in WT mice and not in SAD mice where the I/R liver damage was more severe. We then asked whether the perturbation of HO-1 and BVR response to I/R was also common to other cytoprotective systems as the heat shock proteins (HSPs) that have been shown to functionally cross-talk with NF-κB, to parallel HO-1 expression and to increase in response to I/R stress in other model of ischemic liver damage (54-58). We evaluated HSP70 and 27 expressions 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 (Fig.3). I/R induced increased HSP70 expression in the early phase of hypoxia (4 hrs) in both mouse strains, similarly reducing at 48 hrs hypoxia (Fig.3). In SAD mice, the prolonged I/R stress induced a further increased in HSP70 expression, while WT mice showed marked reduction of HSP70 at 168 hrs hypoxia compared to either normoxic WT mice or 168 hrs hypoxic SAD mice (Fig. 3). HSP27 expression was reduced at 4 and 168 hrs 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 at 168 hrs hypoxia was significant when compared to the corresponding hypoxic WT mouse group (Fig. 3).

Previous in vitro and in vivo studies have shown the hepatoprotective effects of Prx6 in different models of liver injury due to oxidative insults or I/R stress (59-61). Here, we observed a reduction in Prx6 expression in SAD mice under room air condition compared to WT ones (Fig. 3). At 4 hours hypoxia, Prx6 expression was significantly decreased in WT mice, while no changes were present in the corresponding SAD mouse group (Fig.3). At 48 hours 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 either normoxic SAD mice or WT mice at 48 hours hypoxia. After prolonged hypoxia (168 hrs) Prx6 expression was significantly increased only in SAD mice compared to normoxic SAD mouse group, while it was markedly reduced in WT
mice compared to either baseline values or to the other hypoxic WT mouse groups (Fig. 3).
Since previous studies in other model of hepatic ischemic-reperfusion injury have shown possible beneficial effects of phosphodiesterases (PDE) inhibitors (30, 31, 62) we first evaluated the expression of these genes in both mouse strains under normoxia and I/R stress and then we administered the PDE-4 inhibitor Rolipram to SAD mice exposed to prolonged hypoxia compared to untreated SAD mice that developed sickle hepatopathy.

**I/R induced up-regulation of phosphodiesterase-1, 2, 3 and 4 isoforms gene expression**
Since different PDE isoforms are involved in hydrolyzation of cyclic nucleotides with overlapping effects, we evaluated the expression of PDE families -1, -2, -3 that hydrolyze both cAMP and cGMP as substrates but with different affinity and PDE family 4 that hydrolyzes cAMP (36). cAMP and cGMP, acting as second messengers in response to extracellular stimuli, are important in regulation of vascular tone and in modulation of neutrophil chemotaxis (36). In hepatocytes from both mouse strains, the following isoforms were undetectable: PDE3a (111839), PDE4a (115458-69577-39413), PDE4b (106911), PDE4c (34307-110095) and PDE4d (74103-79975). Some differences in PDEs response to hypoxia were observed between WT and SAD mice (Fig. 4). Of interest all PDE4 isoforms were up-regulated in early hypoxia and returned to baseline values in both WT and SAD mice after prolonged hypoxia (Fig. 4c).
Based on these observations, we administrated an inhibitor of PDE4 isoforms (Rolipram) to SAD mice that developed sickle cell hepatopathy corresponding to SAD mice exposed to 168 hrs hypoxia.
PDE-4 inhibitor Rolipram has beneficial effects on sickle cell hepatopathy and modulates cytoprotective systems

SAD mice treated with the PDE-4 inhibitor Rolipram exposed to I/R stress showed significant reduction of (i) liver pathological score; (ii) serum liver transaminases and (iii) liver inflammatory cell infiltrates compared to either untreated SAD mice or hypoxic WT (Table 1, Fig. 5a). We also observed a significant reduction in total neutrophil counts compared to untreated hypoxic SAD mice, suggesting a systemic anti-inflammatory effect of the PDE4 inhibitor (Table 2).

The amelioration of sickle cell related liver damage in SAD mice treated with Rolipram compared to untreated hypoxic SAD mice was also associated with (i) reduced NF-kB p65 activation; (ii) increased in eNOS mRNA levels (iii) increased HO-1 and BVR protein expression associated with marked reduction in HSP70 expression and no changes in HSP27 and Prx6 proteins levels (Fig. 5b, c, d).
DISCUSSION

Here, we show that normoxic SAD mice present a mild chronic hepatopathy associated with increased cellular levels of p-NF-kB p65 in hepatocytes and up-regulation of cytoprotective genes such as eNOS and HO-1 together with increase 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 (17,51,63,64). Whereas, other protective stress-response systems such as the molecular chaperones HSP70, -27 and the endogenous anti-oxidant Prx6 (59-61) were similarly expressed in both mouse strains under steady state, suggesting that HSPs and Prx6 are not involved in cell protection against chronic sickle cell related mouse liver damage.

In SAD mice exposed to I/R stress protocol to induce liver damage compatible with sickle cell hepatopathy we study the molecular mechanisms involved in the liver damage of SCD. The development of severe sickle cell hepatopathy was characterized by temporal differences in NF-kB p65 activation in SAD mice compared to WT ones in response to I/R stress. Previous studies in other models of liver injury have shown the important role of NF-kB p65 in signal transduction towards cytoprotective genes as eNOS, iNOS and HO-1 (48, 65-68). In SAD mice, we observed a lack in hypoxia induced eNOS expression in the early phase of I/R stress compared to 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 (69-71). In SAD mice the unbalance between iNOS/eNOS expression in response to I/R stress compared to WT mice may unfavorable affect the NO bioavailability, contributing to the liver microcirculation dysfunction involved in the development of sickle hepatopathy (Fig. 3). In addition, the response of other cytoprotective systems as HO-1, BVR, HSPs and Prx6 to I/R stress also differs in SAD mice compared to WT ones, representing a new additional factor possibly increasing SAD liver susceptibility to I/R stress. The lack in HO-1 and BVR up-
regulation in response to I/R stress observed in SAD mice might contribute to the development of a more severe cellular damage beside the biphasic increased of HSP70 that seems not to be enough to counteract the sickle cell related acute organ injury (Fig. 5). In SAD mice, the small HSP27 and Prx6 expression were both increased after prolonged hypoxia when compared to WT mice, which showed a significant reduced expression of both proteins. Although HSP27 and Prx6 are members of different functional groups: the anti-oxidant systems and the molecular chaperones, they are both induced in response to oxidative stress in other cell types and their low expression increased cell susceptibility to oxidative stress (60, 61, 72, 73). These data supported by the literature evidences indicate a possible novel hepatoprotective role of HSP27 and Prx6 in SAD mice exposed to prolonged I/R stress.

Previous studies in various model of I/R liver injury have shown beneficial effects of phosphodiesterases' inhibitors (30-34, 74). Here, we showed the up-regulation of PDE1, 2, 3, 4 in the early phase of hypoxia (4 hours) in both mouse strains indicating their involvement in the cellular response to the I/R cell injury. The block of PDEs-4 by Rolipram allowed SAD mice to survive to prolonged hypoxia and ameliorated the sickle cell related liver injury as supported by (i) the low liver pathological score; (ii) the market decreased in local and systemic cell inflammatory response; (iii) the up-regulation of eNOS gene expression balancing iNOS expression; (iv) the increased HO-1/BVR expression with reduced HSP70 levels. These data suggest that in SAD mice the inhibition of PDE4s 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-kB 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 that may possible represent targets for the development of new therapeutic strategies.
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AUTHORSHIP AND DISCLOSURES
LDF and YB obtained institutional review board and consent, designed and conducted studies and prepared the manuscript, AS performed experiments and analysed data, GM, AS and EA performed pathological preparation and RNA analysis. OSP, RC and OO contributed to the discussion and manuscript preparation. AJ and CL performed pathological studies, analysed data and contributed to manuscript preparation.
The Authors reported no potential conflicts of interest.
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# Table 1

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

<table>
<thead>
<tr>
<th>Pathological score</th>
<th>WT mice</th>
<th>SAD mice</th>
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<tr>
<td></td>
<td>Normoxic</td>
<td>Hypoxic 4 hr</td>
</tr>
<tr>
<td>Inflammatory cell infiltration</td>
<td>0 (0)</td>
<td>0.5 ± 0.02 (0)</td>
</tr>
<tr>
<td>Thrombi (% of section examined)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Inflammatory cell infiltration:
- 0: 0-10 cells per field at magnification × 400
- +: 11-50 cells per field at magnification × 400
- ++: 51-100 cells per field at magnification × 400
- +++: >100 cells per field at magnification × 400

Pathological scores:
1: no hepatocellular damage
2: mild injury characterized by cytoplasmic vacuolization and fixed nuclear pyknosis.
3: moderate injury with cytoplasmic vacuolization and blunting of intercellular spaces.
4: severe injury with loss of hepatocyte architecture, disintegration of hepatocellular plates, hypercoastophilia and migration of neutrophils.

Data are presented as mean ± SD (n of experiments); *P < 0.05 vs normoxic; †P < 0.05 vs WT mice; *P < 0.05 vs wild-type.
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<th>WT mice</th>
<th>SAD mice</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Hypoxia</td>
</tr>
<tr>
<td></td>
<td>4 hrs</td>
<td>48 hrs</td>
</tr>
<tr>
<td></td>
<td>168 hrs</td>
<td>168 hrs</td>
</tr>
<tr>
<td></td>
<td>±0</td>
<td>±0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hct (%)</td>
<td>48.3±0.84</td>
<td>48.8±1.8</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>14.4±0.8</td>
<td>15.1±0.4</td>
</tr>
<tr>
<td>Retic (µL)</td>
<td>4.5±1.9</td>
<td>4.2±0.2</td>
</tr>
<tr>
<td>RBCs (x10^6)</td>
<td>1.096±0.002</td>
<td>1.059±0.002</td>
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</tbody>
</table>
| Data are presented as means ± SD of experiments. Hct: hematocrit; Hb: hemoglobin; RBCs: red blood cells; Retic: reticulocytes; AST: aspartate aminotransferase; ALT: alanine aminotransferase; *P<0.05 vs baseline; *P=0.05 vs wild-type normal mice; ±0 = 0.05 vs 168 hrs hypoxia untreated SAD mice; ±0 = 0.05 vs wild-type 168 hrs hypoxia.
FIGURE LEGENDS

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, 168 hrs) followed by 2 hours 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 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. (b) Immunoblot analysis with specific anti phospho-NF-kB p65 and anti NF-kB p65 antibody of hepatocytes from WT and SAD mice under normoxia (time 0) and hypoxia (4, 48, 168 hrs) followed by 2 hrs reoxygenation. Shown is a representative experiment of 6 performed with similar results.

Figure 2. Quantitative RT-PCR expression profile of eNOS (endothelial NO synthase), iNOS (inducible NO synthase), heme oxygenase-1 (HO-1), in laser-captured hepatocytes from WT mice and SAD mice under normoxia (time 0) and hypoxia (4, 48, 168 hrs) followed by 2 hours 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 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.

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, 168 hrs) followed by 2 hrs reoxygenation. Shown is a representative experiment of 6 performed with similar results. Expression of actin was used as a protein loading control.
Figure 4. (A,B,C) Effects of ischemic/reperfusion protocol (I/R) on phosphodiesterase (PDE) 1, 2, 3, 4 isoforms gene expression 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 hrs hypoxia followed by 2 hours 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.

Figure 5. (A) The panel shows the representative example of SAD mouse liver after 168 hrs hypoxia with or without the PDE-4 inhibitor Rolipram (see also Table 1). (B) Immunoblot analysis with specific anti phospho-NF-kB p65 and anti-NF-kB p65 antibody of livers from SAD mice exposed to 168 hours hypoxia followed by 2 hours reoxygenation with and without the PDE-4 inhibitor Rolipram (SAD+R). Expression of actin was used as loading control protein. Shown is a representative experiment of 6 performed with similar results. (C) Quantitative RT-PCR expression profile of eNOS (endothelial NO synthase), iNOS (inducible NO synthase), heme oxygenase-1 (HO-1), in laser captured hepatocytes from SAD mice exposed to 168 hrs hypoxia with or without Rolipram (168+R) followed by 2 hours 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 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. (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 exposed to hypoxia with or without Rolipram (SAD+R) followed by 2 hrs reoxygenation. Shown is a representative experiment of 6 performed with similar results.
Fig. 1

a. Relative NFκB mRNA (log)

b. Western Blot of p-NFκB p65 (Ser 536), NFκB p65, and Actin under Hypoxia conditions in WT and SAD cells.
Fig. 2

**eNos**

![Graph showing relative eNOS mRNA expression over time (0-168 hours) for WT and SAD groups.](image)

**iNos**

![Graph showing relative iNOS mRNA expression over time (0-168 hours) for WT and SAD groups.](image)

**HO-1**

![Graph showing relative HO-1 mRNA expression over time (0-168 hours) for WT and SAD groups.](image)
### ONLINE SUPPLEMENTARY TABLE 1

**LIST OF GENES STUDIED BY QUANTITATIVE RT-PCR AND OF THE PRIMERS USED.**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene code</th>
<th>Primer sequence (5’-3’)[c]</th>
<th>Size[d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rela, nuclear factor NF-kappa B, p65 subunit</td>
<td>NM_009405</td>
<td>F- GCTCCTGTTTCAGCTTCATG</td>
<td>101</td>
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<tr>
<td></td>
<td>NM_010927</td>
<td>R- CGGTCGCACTTCATG</td>
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<tr>
<td></td>
<td></td>
<td>F- ACAATCAAGGCGCCTCACC</td>
<td>86</td>
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<tr>
<td></td>
<td></td>
<td>R- CAGCGTACCGGAGTACGTG</td>
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<tr>
<td>Nos2, nitric oxide synthase, inducible (iNOS)</td>
<td>NM_008713</td>
<td>F- GGTATCCCCGGGTCTCGT</td>
<td>76</td>
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<tr>
<td>Nos3, nitric oxide synthase, endothelial (eNOS)</td>
<td>NM_010442</td>
<td>R- GTCACCCCAACACAGCTGCC</td>
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<tr>
<td>Hmox1, heme oxygenase 1 (HO-1)</td>
<td>NM_008713</td>
<td>F- AGAGGCTAAGACGGCCTGCC</td>
<td>101</td>
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<tr>
<td></td>
<td></td>
<td>R- AGCCGATCTGTGAGGACTTC</td>
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<td>Pde1a, 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000102651</td>
<td>F- TCTTTAAGAAGCTGCGACACAGA</td>
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<td></td>
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<td>R- CAATGCGCTAGAACTTTGGT</td>
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<td>Pde1b, 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000023132</td>
<td>F- AGGCGCTATTCTCTCTGCTCA</td>
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<tr>
<td>Pde1c, 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000044505</td>
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<td>Pde1d, 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000114326</td>
<td>F- AGGCTCTGATGGCTCACTT</td>
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<tr>
<td></td>
<td></td>
<td>R- TCCGATGCTCTGCCACAAAG</td>
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<tr>
<td>Pde2a, 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST0000032889</td>
<td>F- CATGCGGACACTCATCAC</td>
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<td>Pde2b, 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST0000098241</td>
<td>F- GGCCTGACCTCTTCTGAG</td>
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<td>Pde3a, 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000111839</td>
<td>F- CACCCGCTGCCACAAATG</td>
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<td>Pde3b, 3',5' cyclic nucleotide phosphodiesterase</td>
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<td>ENSMUST00000115458</td>
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<td>ENSMUST00000039413</td>
<td>F- GCCCTAGAGGACGAGTCT</td>
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<td>Pde4c, 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000039413</td>
<td>F- TGGCCCTAGAGGACGAGTCT</td>
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<tr>
<td>Pde4d, (PDE4B1) 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000106911</td>
<td>F- CAGGAGGACGGTCTCTCAC</td>
<td>71</td>
</tr>
<tr>
<td>Pde4e, (PDE4B4) 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000106908</td>
<td>F- ATATACATCTGGCGATCC</td>
<td>101</td>
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<td>Pde4f, (PDE4B2) 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000097950</td>
<td>F- TTGGAAGCGAGGATCGGC</td>
<td>271</td>
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<td>Pde4g, (PDE4B5) 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000069577</td>
<td>F- CGGCTGTTGCTGACAGACA</td>
<td>226</td>
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<td>Pde4h, (PDE4B1) 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST000000097950</td>
<td>F- TGGCCCTAGAGGACGAGTCT</td>
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<tr>
<td>Pde4i, (PDE4B3) 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000097950</td>
<td>R- CTTCTGAGGACGAGTCCG</td>
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<tr>
<td>Pde4j, (PDE4B5) 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST000000097950</td>
<td>R- TGGCCCTAGAGGACGAGTCT</td>
<td></td>
</tr>
<tr>
<td>Pde4k, (PDE4B1) 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST00000097950</td>
<td>R- TGGCCCTAGAGGACGAGTCT</td>
<td></td>
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<tr>
<td>Pde4l, (PDE4B3) 3',5' cyclic nucleotide phosphodiesterase</td>
<td>ENSMUST000000097950</td>
<td>R- TGGCCCTAGAGGACGAGTCT</td>
<td></td>
</tr>
</tbody>
</table>

[a] includes official symbols and names; in parentheses are the gene aliases cited in text
[b] DNA reference sequence or Ensembl transcript for phosphodiesterases (www.ensembl.org/Mus_musculus/)
[c] F: forward; R: reverse
[d] length of the PCR product in base pair