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

Angela Siciliano,[1] Giorgio Malpeli,[2] Orah S. Platt,[3] Christophe Lebouef,[4,5,6] Anne Janin,[4,5,6,] Aldo Scarpa,[2] Oliviero Olivieri,[1] Eliana Amato,[2] Roberto Corrocher,[1] Yves Beuzard,[7] and Lucia De Franceschi[1]

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

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Supplemetary Design and Methods

Liver histopathology

The pathological score is defined as follows: 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; and 4, *severe necrosis* with loss of hepatic architecture, disintegration of hepatic chords, hemorrhage and neutrophil infiltration. We also evaluated the inflammatory cell infiltrate and the presence of thrombi. Morphological analyses were 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 was less than 5%.

Molecular studies by quantitative reverse-transcription polymerase chain reaction analysis of hepatocytes obtained by laser capture microdissection

The cDNA of cells isolated by laser capture microdissection (LCM) was pre-amplified by 14 polymerase chain reaction (PCR) cycles (each cycle consisted of 15 s at 95°C and 240 s at 60°C) in a solution including 0.05X Taqman probes or 50 nM forward and reverse oligonucleotide primers, 5 μL of cDNA and 1x Taqman PreAmp Master Mix (Applied Biosystems). The pre-amplified solution was diluted 1/20 and 5 μL of the diluted solution were used as a template for subsequent quantitative reverse-transcription PCR (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 were performed in a final volume of 20 μL. Thermal cycling included in all cases an initial incubation at 95°C for 10 min then 40 PCR cycles (15 s at 95 °C and 60 s at 60°C). Samples were analyzed in triplicate on an ABI Prism 7900 SDS instrument (Applied Biosystems). The oligonucleotide primers used in qPCR are shown in *Online Supplementary Table S1*. 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 *rRNA18S* (Hs99999901_s1) as endogenous references. Data were analyzed as indicated in User Bulletin #2 (Applied Biosystems).

Antibodies used in immunoblot analysis

Gels were transferred to nitrocellulose membranes for immunoblot analysis with specific antibodies against nuclear factor-κB p65 (NF-κB p65, clone C22B4, Cell Signaling), phospho-nuclear factor-κB p65 (p-NF-κB 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 actin (Sigma Chemical Co., St Louis, MO; USA). Actin was used as the loading control.

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Functional link between heme oxygenase-1 and biliverdin reductase

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Functional cross-talk of heat shock proteins with nuclear factor-κB, parallel heme oxygenase-1 expression and increase in response to ischemic/reperfusion stress in other models of ischemic liver damage

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The hepatoprotective effects of Prx6 in different models of liver injury due to oxidative insults or ischemic/reperfusion stress

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Online Supplementary Table S1. List of genes studied by quantitative rt-pcr and of the primers used.

Gene name[a]	Gene code[b]	Primer sequence (5'-3')[c]	Size[d]
Rela, nuclear factor NF-kappa B, p65 subunit	NM_009045	F- GCTCCTGTTCGAGTCTCCATG R- CGGTGGCGATCATCTGTGT	101
Nos2, nitric oxide synthase, inducible (iNOS)	NM_010927	F- AACATCAGGTCGGCCATCAC R- CAGCGTACCGGATGAGCTGT	86
Nos3, nitric oxide synthase, endothelial (eNOS)	NM_008713	F- TTGATCCCCGGGTCCTGT R- GTCACCACCAACACCAGTGC	76
Hmox1, heme oxygenase 1 (HO-1)	NM_010442	F- AGAGGCTAAGACCGCCTTCC R- ACGCCATCTGTGAGGGACTC	101
Pde1a, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000102651	F- TCTTTAGAAGACTGCTGGACACAGA R- CAATGCTGCGAAACTTTGGTT	156
Pde1b, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000023132	F- AGGCCCTATCTCTTCTGCTTCA R- CACCCTGGCGGAAGAACTC	111
Pde1c, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000044505	F- GCTCACCTGCTCCGAGCA R- GGAGGCTTGATGACTGGCAA	61
Pde1c, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000114326	F- AGCGTTCTCATGGCTCACCT R- TCCGTAGTCTCCTGGCAAGG	71
Pde2a, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000032889	F- CATGCGGCCACTCCATC R- CCCGCGGCTCAGCC	66
Pde2a, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000098241	F- GGGCTTGCACCCTTTTCAG R- CCGCCGTTCCCAAATGT	96
Pde3a, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000111839	F- CCACGAGGATCCCAGGAAA R- TTCCACATCATGTGGTTCTGC	91
Pde3a, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000043259	F- CCTCAGGCGGTGCTATACAAC R- AAGTGCTTGAATTCCACGTGG	131
Pde3b, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000032909	F- TTCAATGCCAAGGCCAATG R- ATTTGATGCACACCTGGCAG	91
Pde4a, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000003395	F- TGCCAGCCCAGAGATAAGCT R- CCTTTTAAACTGGTCCCACCAG	101
Pde4a, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000115458	F- AGCAGTAGGCGCTTGGAGG R- ACGGATGAGTTCCTGGACATAG	191
Pde4a, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000069577	F- CTCTATCGCTCAGACAGCGAC R- GTGTGGCCTTGCAGACAGA	226
Pde4a, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000039413	F- GCCCCTAGGACCGGAGTC R- TGCCTCCAAGCTGACACATCT	91
Pde4b, (PDE4B1) 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000106911	F- CAGAGGAGCTGTTTCCCACA R- ATTATCATCTGCGGTACCG	71
Pde4b, (PDE4B4) 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000106908	F- CGCAGGGAGTCGTTCCTCTA R- TCATCGCCGTGTTGCTCA	101
Pde4b, (PDE4B2) 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000097950	F- TTGGAAGCAAGGAGTCGGC R- CATCCTTTTGAACTTGTTTGAAGC	271
Pde4b, (PDE4B5) 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000106901	F- TTCCTGATCCCCCTGGAGAC R- TCCTTTTGAACTTGATATAACCCCA	141
Pde4b, (PDE4B3) 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000106904	F- GCTCTGCACTAGGTGTTGGGA R- AAAAAGGCACACAGGTTGGC	111
Pde4c, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000034307	F- ACTCTGACCGCATCCAGCA R- AGGCTGTGTGTTTGTCGCAC	81
Pde4c, 3',5' cyclic nucleotide phosphodiesterase	ENSMUST00000110095	F- AACTACTCTGACCGCATCCAGG R- TGTGTGTTTGTCGCACATGG	183

[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] lenght of the pcr product in base pair