Annexin A6 is critical to maintain glucose homeostasis and survival during liver regeneration in mice

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SUPPLEMENTARY MATERIAL AND METHODS

SNAT4 antibody production

A rabbit polyclonal antibody was raised against the N-terminal region (aa 1-80) of mouse SNAT4 bound to glutathione-S-transferase (GST) as described previously [1]. The recombinant protein was produced in *Escherichia coli* BL21(DE3) pLysS (Promega) and affinity purified with Glutathione-sepharose 4B beads (GE Healthcare). The SNAT4 antibody was validated by western blot analysis, recognizing endogenous (mouse) and recombinant (mouse) SNAT4 in liver samples and cell lysates ectopically expressing SNAT4-GFP.

Western blot analysis

Crude liver lysates were prepared in lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.5 and protease inhibitors) and equal amounts of protein, as determined by Bradford assay [2], were separated by 10% SDS-PAGE and transferred to a Hybridation Nitrocellulose membrane (Millipore). After blocking in 5% skimmed milk, western blot was performed using rabbit anti-AnxA6 serum [1], goat anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Genescript, A00191), rabbit anti-green fluorescent protein (GFP) antibody (Abcam, ab290), mouse anti-β-actin (MP Biomedicals) and rabbit anti-SNAT4 serum (see above). Appropriate peroxidase-conjugated secondary antibodies (BioRad) and enhanced chemiluminescence detection (Amersham Biosciences) for band detection were used. The intensity of AnxA6 or SNAT4 signals was quantified using ImageJ [3] and results were normalized to GAPDH.

Recombinant adeno-associated viral particle (rAAV)-AnxA6 production and administration

To confer liver-specific expression, rat AnxA6 and GFP cDNAs were cloned into the single strand AAV2-EalbAAT-WPRE plasmid, which contains an expression cassette flanked by two inverted terminal repeats from AAV2, the human α-1-antitrypsin promoter with regulatory sequences from

the human albumin enhancer (EalbAAT) and the bovine growth hormone polyadenylation sequence [4]. rAAV2/8 viruses were produced with envelope proteins of serotype 8 [5] in human embryonic kidney (HEK)-293 cells and purified by polyethylene glycol precipitation and iodixanol-gradient centrifugation [6]. Nine 8-week-old WT and AnxA6$^{-/-}$ mice were injected via the tail-vein with 1 x 10^{11} cg of rAAV-AnxA6 or rAAV-GFP vectors. Experiments were performed 15 days after infection.

RNA extraction and quantitative Real-Time PCR

Total RNA was prepared from mice liver using RNeasy Lipid Tissue Mini Kit (Qiagen) in accordance with the manufacturer's protocol. 1 µg RNA from each sample was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Bioscience). For PCR analysis, a final volume of 20 µl real-time PCR Brilliant SYBRGreen QPCR Master Mix (Agilent Technologies), containing 10 µl of 1:20 diluted cDNA as a template and specific primers for *cJun*, *cFos*, *Ccnd1*, *Slc38a2*, *Slc38a4*, *Pck1*, *Fbp1*, *G6pc1*, *Gpt1*, *Gpt2* and *Tbp* (see Supplementary Table 1), were analysed following a standard PCR amplification protocol (10 min at 95ºC, 45 cycles of 30 sec at 95ºC, 15 sec at 60ºC and 30 sec at 72ºC, 10 sec at 95ºC and 60 sec at 65ºC) and the LightCycler system (Roche Diagnostics), according to manufacturer's instructions. Relative mRNA expression was normalised to the housekeeper *Tbp* levels using the ΔΔCT method [7].

Biochemical analysis

Blood was collected by intracardial punction into BD Microtainer tubes. Plasma levels of alanine aminotransferase (ALAT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), albumin and total protein were analysed according to manufacturer's instructions (Adiva 1650, Bayer). Plasma was prepared and free cholesterol, high- and low-density lipoproteins (HDL, LDL), triacylglycerides (TAG) and free fatty acids were measured by colorimetric and spectrometric techniques (BioSystems).

Blood glucose levels were determined from blood obtained by a small incision in the mouse tail using a glucometer (Glucocard G+ meter set, Arkray).

Insulin (US Mouse Insulin ELISA, Mercodia) plasma levels were determined by ELISA according to manufacturer's instructions.

For the determination of liver glycogen levels, 200 mg liver sample was homogenized in 1 ml 30% KOH at 100ºC for 10 min. Samples were left to cool at room temperature and 2 ml of ethanol was added, followed by a 24 h incubation at -20°C. Samples were then centrifuged at 2,000 g for 15 min at 4ºC and the supernatant was discarded. The pellet was resuspended in 3 ml of 1:2 distilled water and ethanol (v/v) at 4ºC. Samples were then centrifuged at 2,000 g for 15 min at 4ºC, the pellet was resuspended in 1 ml 5 N H_2SO_4 and incubated for 2 h at 100ºC. Finally, samples were neutralized with 1N NaOH and phenolphthalein (Fluka) as pH indicator, and the glucose levels corresponding to liver glycogen were determined using the Glucose assay kit (Sigma) according to manufacturer's instructions.

For the determination of blood ketone bodies, blood was collected by intracardiac punction in BD Microcontainer tubes. 5 µl of blood sample was analysed with Ketone bodies kit (Sigma) following the manufacturer's instructions.

For liver TAG quantification, liver samples were homogenised in RiboTubes with buffer (10 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.5) and protease inhibitors. The samples were disaggregated in a bullet blender, centrifuged at 10,000 rpm for 6 min at 4ºC and quantified [2]. 40 µg liver protein was then analysed using the TAG kit (BioSystems).

Plasma and liver amino acid levels

Standards, plasma and homogenized liver samples were mixed with the internal standard solution and methanol/0.1% formic acid to precipitate the proteins. Then, the samples were centrifuged at 6,000 x g for 10 minutes. Amino acids were derivatized in borate buffer (pH = 8.8) using the AccQ·Tag Ultra Derivatization Kit (Waters). Plasma and liver amino acids were then analysed by ultra performance liquid chromatography (UPLC) coupled to tandem mass spectrometry, as previously reported [8]. Briefly, amino acids were separated in a Waters ACQUITY UPLC H-class chromatograph and quantified with a Waters Xevo TQD triple-quadrupole mass spectrometer using positive electrospray ionization conditions in the MRM mode as described.

Isolation of primary hepatocytes and in vitro *glucose production*

Primary hepatocytes were isolated by in situ perfusion of collagenase (from *Clostridium histolyticum*, Sigma) as described previously [9]. To determine gluconeogenic capacity, 10^6 primary hepatocytes were plated in 6-well plates and grown overnight in DMEM, 2% FCS and

200 nM dexamethasone (Sigma). Then primary hepatocytes were starved for 6 h in DMEM without glucose, and incubated with 2 mM pyruvate or 20 mM glutamine, lactate and alanine for 24 h. The glucose production in the media was analyzed using a Glucose GO assay kit (Sigma) according to manufacturer's instructions.

SUPPLEMENTARY FIGURES

Supplementary Figure 1. Hepatic functions in WT and AnxA6$^{-/-}$ mice.

A. Plasma AST, ALAT, LDH, albumin and total protein levels in WT (n=4) and AnxA6$^{-/-}$ (n=4) mice.

B. Plasma levels of free cholesterol, TAGs and free fatty acids in WT (n=4) and AnxA6$^{-/-}$ (n=4) mice.

C. Plasma HDL and LDL levels in WT (n=4) and AnxA6$^{-/-}$ (n=4) mice.

D. Plasma ALAT levels in WT (n=6) and AnxA6$^{-/-}$ (n=6) mice after PHx.

E. Plasma AST levels in WT (n=6) and AnxA6$^{-/-}$ (n=6) mice after PHx.

Supplementary Figure 2. Blood glucose levels and SNAT4 localization in rAAV-AnxA6 infected AnxA6$^{-/-}$ mice.

A. Blood glucose levels in rAAV-AnxA6 infected AnxA6$^{-/-}$ mice (n=5) after PHx (0-72 h).

B. Representative confocal laser scanning microscopy of rAAV-AnxA6 infected AnxA6$^{-/-}$ liver sections at 0, 12, 24 and 48 h after PHx stained for SNAT4 (green), phalloidin (actin, red) and DAPI (nucleus, blue). Scale bar, 10 μm.

C. Quantification of SNAT4 intensity ratio between sinusoidal plasma membrane and cytoplasm intensities of rAAV-AnxA6 infected AnxA6$^{-/-}$ liver sections (n=4) at 0, 12, 24 and 48 h after PHx.

Supplementary Figure 3. Amino acid levels in plasma after PHx in WT and AnxA6$^{-/-}$ mice (0-48 h, n=4 each group).

Supplementary Figure 4. Amino acid levels in liver after PHx in WT and AnxA6$^{-/-}$ mice (0-48 h, n=4 each group).

Supplementary Figure 5. Branched-chain amino acid (BCAA) levels and hepatic expression of gluconeogenic genes after PHx in WT and AnxA6$^{-/-}$ mice.

A and B. BCAA plasma and liver levels in WT and AnxA6$^{-/-}$ mice after PHx (0-48 h, n=4 each group).

C and D. Relative mRNA expression levels of fructose-1,6-bisphosphatase (*Fbp1*) and glucose-6-phosphatase (*G6pc1*) determined by real-time PCR in WT and AnxA6$^{-/-}$ mice livers after PHx (n=5).

SUPPLEMENTARY TABLE

Supplementary table 1. Mouse-specific primer sequences for quantitative PCR.

Gene (protein)	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
cJun (cJun)	CGGACTCCGGACTGTTCATC	TTTGCAAAAGTTCGCTCCCG
cFos (cFos)	GGGAATGGTGAAGACCGTGT	GGGAATGGTGAAGACCGTGT
Ccnd1 (Cyclin D1)	GAGCTGCTGCAAATGGAACT	ATGGAGGGTGGGTTGGAAAT
Slc38a2 (SNAT2)	TGAAAAGCCATTATGCCGACG	CCCACAATCGCATTGCTCAG
Slc38a4 (SNAT4)	CAGAAAGGCGGGAAAGGGCT	TGTTCATGGCGTCCTTGTCG
Pck1 (PEPCK1)	GTCTGGCTAAGGAGGAAGGG	GCCAGGAGCAACTCCAAAAA
Fbp1 (FbisPase1)	ACCTGCCTGCACCTTTAGTC	TTGGTTGAGCCAGCGATACC
G6pc1 (G6pc)	TTACCAAGACTCCCAGGACTG	GAGCTGTTGCTGTAGTAGTCG
Gpt1 (ALAT1)	TCCAGGCTTCAAGGAATGGAC	CAAGGCACGTTGCACGATG
Gpt2 (ALAT2)	CAGACCCAGACAACATTTACCTG	CGCGGAGTACAAGGGATACTG
Tbp	CACCCCTTGTACCCTTCAC	TTCACTCTTGGCTCCTGTGC

SUPPLEMENTARY REFERENCES

[1] Grewal T, Heeren J, Mewawala D, Schnitgerhans T, Wendt D, Salomon G, et al. Annexin VI stimulates endocytosis and is involved in the trafficking of low density lipoprotein to the prelysosomal compartment. J Biol Chem 2000;275:33806-33813.

[2] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254.

[3] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature methods 2012;9:671-675.

[4] Unzu C, Sampedro A, Mauleon I, Alegre M, Beattie SG, de Salamanca RE, et al. Sustained enzymatic correction by rAAV-mediated liver gene therapy protects against induced motor neuropathy in acute porphyria mice. Mol Ther 2011;19:243-250.

[5] Sarkar R, Tetreault R, Gao G, Wang L, Bell P, Chandler R, et al. Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. Blood 2004;103:1253-1260.

[6] Hermens WT, ter Brake O, Dijkhuizen PA, Sonnemans MA, Grimm D, Kleinschmidt JA, et al. Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. Hum Gene Ther 1999;10:1885-1891.

[7] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-408.

[8] Casado M, Sierra C, Batllori M, Artuch R, Ormazabal A. A targeted metabolomic procedure for amino acid analysis in different biological specimens by ultra-high-performance liquid chromatography-tandem mass spectrometry. Metabolomics 2018;14:76.

[9] Bosch M, Mari M, Herms A, Fernandez A, Fajardo A, Kassan A, et al. Caveolin-1 deficiency causes cholesterol-dependent mitochondrial dysfunction and apoptotic susceptibility. Curr Biol 2011;21:681-686.