Research Article

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Model-guided identification of a therapeutic strategy to reduce hyperammonemia in liver diseases

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Background & Aims: Recently, spatial-temporal/metabolic mathematical models have been established that allow the simulation of metabolic processes in tissues. We applied these models to decipher ammonia detoxification mechanisms in the liver.
Methods: An integrated metabolic-spatial-temporal model was used to generate hypotheses of ammonia metabolism. Predicted mechanisms were validated using time-resolved analyses of nitrogen metabolism, activity analyses, immunostaining and gene expression after induction of liver damage in mice. Moreover, blood from the portal vein, liver vein and mixed venous blood was analyzed in a time dependent manner.
Results: Modeling revealed an underestimation of ammonia consumption after liver damage when only the currently established mechanisms of ammonia detoxification were simulated. By iterative cycles of modeling and experiments, the reductive amidation of alpha-ketoglutarate (α-KG) via glutamate dehydrogenase (GDH) was identified as the lacking component. GDH is released from damaged hepatocytes into the blood where it consumes ammonia to generate glutamate, thereby providing systemic protection against hyperammonemia. This mechanism was exploited therapeutically in a mouse model of hyperammonemia by injecting GDH together with optimized doses of cofactors. Intravenous injection of GDH (720 U/kg), α-KG (280 mg/kg) and NADPH (180 mg/kg) reduced the elevated blood ammonia concentrations (>200 μM) to levels close to normal within only 15 min.
Conclusion: If successfully translated to patients the GDH-based therapy might provide a less aggressive therapeutic alternative for patients with severe hyperammonemia.

Keywords: Systems biology; Spatio-temporal model; Ammonia; Liver damage; Liver regeneration.
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Abbreviations: CCl_4, carbon tetrachloride; GDH, glutamate dehydrogenase; AOA, aminooxy acetate; ALT, alanine transaminase; AST, aspartate transaminase; α-KG, alpha-ketoglutarate; PDAC, 2,6-pyridinedicarboxylic acid.

Introduction

Recent developments have strongly improved our capability to generate information at multiple spatial and temporal scales [1,2]. However, research on disease pathogenesis is hampered by the difficulty to understand the orchestration of individual components. Here, mathematical models help to formalize relations between components, simulate their interplay, and to study processes that are too complex to be understood intuitively [1]. This is particularly important when studying the pathophysiology of metabolic liver diseases, where due to zonation different metabolic processes take place in pericentral and periportal hepatocytes [3]. To be able to investigate such complex processes we recently established a technique of integrated metabolic spatial-temporal modeling (IM) [4]. These IM integrate conventional metabolic models into spatial-temporal models of the liver lobule [1,4,5]. The present study was motivated by the IM predictions, which proposed that the conventional mechanisms where

ammonia is metabolized by urea cycle enzymes in the periportal compartments of the liver lobules and by glutamine synthetase (GS) reaction in the pericentral compartments (Supplementary Fig. 1) failed to explain the experimental findings [4]. The IM was applied to an experimental scenario, where the entire pericentral and a part of the periportal compartment of the liver lobules were destroyed by a single high dose of the hepatotoxic compound carbon tetrachloride (CCl_4). This leads to compromised nitrogen metabolism and hyperammonemia. In the present study, we performed a series of new experiments accompanied by simulations with novel models to explore the mechanism responsible for the observed discrepancy. Experimentally, the time-resolved analysis of metabolites and metabolic activities after CCl_4 intoxication offers good conditions to study ammonia detoxification and possible compensatory mechanisms during the damage and regeneration process. Time-resolved analysis of metabolites was performed in the portal vein and heart blood, representing the 'liver inflow', and in the liver vein as 'liver outflow'. These analyses allowed a precise experimental validation of model predictions. Finally, iterative cycles of modeling and experimental validation allowed the identification of a so far unrecognized mechanism of ammonia detoxification. Importantly, this mechanism could be exploited therapeutically to reduce elevated blood ammonia concentrations close to normal levels by intravenous injection of glutamate dehydrogenase (GDH; 720 U/kg) and its cofactors alpha-ketoglutarate (α-KG; 280 mg/kg) as well as NADPH (180 mg/kg). This example illustrates how concrete therapies can be derived by model guided experimental strategies.

Materials and methods

A detailed description of materials and methods is provided in the Supplementary materials. Male C57BL/6N 10–12 weeks old mice were used (Charles River, Sulzfeld, Germany). Acute liver damage was induced by intraperitoneal injection of 1.6 g/kg CCl_4, unless other doses are indicated. Blood was taken from mice under anesthesia from the portal and hepatic veins, as well as the right heart chamber, and plasma was separated. Liver tissue samples were collected from defined anatomical positions for histopathology, immunohistochemistry, enzyme activity assays, gene array and qRT-PCR analyses. The dead cell area was quantified in hematoxylin and eosin stained tissue sections using Cell^M software (Olympus, Hamburg, Germany). Whole-genome analysis of gene expression in mouse liver tissue was performed in control as well as after CCl_4 intoxication with Affymetrix gene arrays. The latter techniques are described fully in the Supplementary materials and methods. The analysis of ammonia and further metabolites was performed using commercially available kits. Concentrations of amino acids and organic acids in liver tissue were measured in duplicate using GC-MS. GS, GDH and transaminases activity assays were performed photometrically as described in the Supplementary materials and methods. $NADP^+$ and NADPH were analyzed by LC-MS. Mouse hepatocytes were isolated by a two-step EGTA/collagenase perfusion technique and either used directly in suspension or cultivated in collagen sandwiches (Supplementary materials and methods). For the mathematical modeling of ammonia and the related metabolites the integrated metabolic, spatio-temporal model was applied [4,5]. In addition, the IM was replaced by a set of novel models that include further reactions and the blood compartment of the liver (Supplementary materials and methods). Statistical analysis was done with SPSS software as described in the Supplementary materials.

Results

An integrated spatial-temporal-metabolic model suggests a so far unrecognized mechanism of ammonia detoxification

The detoxification process in healthy, damaged and regenerating livers was simulated using a recently established integrated metabolic IM [4]. To compare the simulated metabolite concentrations with the *in vivo* situation, an experiment was performed in which blood was collected from the portal vein (representing 85% of the 'liver inflow'), the heart (representing 15% of the 'liver inflow'), and the hepatic vein (representing the 'liver outflow') in a time-resolved manner after CCl_4 injection (Fig. 1A; Supplementary Fig. 2). The result shows that ammonia is detoxified during its passage through the liver as illustrated by the difference in ammonia concentrations between the portal vein and the hepatic vein in the control mice (Fig. 1B). This detoxification process is compromised after liver damage, particularly on days 1 and 2. Surprisingly, the IM model predicted higher ammonia concentrations than those experimentally observed, particularly on day 1 (Fig. 1C; see the video in the Supplementary data). Analyses of heart blood demonstrate the contribution of the extrahepatic compartment, which includes brain, muscles, kidneys and blood, to ammonia detoxification between days 1 and 4 after the induction of liver damage. However, this extrahepatic contribution is small compared to detoxification by the liver (Supplementary Figs. 2–8). In addition to the time-resolved study, similar experiments were also performed in a dose dependent manner on day 1 after CCl_4 administration when the discrepancy between simulated and measured ammonia was maximal. For this purpose, doses ranging between 10.9 and 1600 mg/kg CCl_4 were tested, resulting in a concentration dependent increase in the dead cell area, with only the highest dose causing damage to the entire CYP2E1 positive pericentral region of the liver lobule (Fig. 1D; Supplementary Fig. 9A, B). Destruction of the GS positive area occurred in with doses ranging between 38.1 and 132.4 mg/kg (Fig. 1D, E; Supplementary Fig. 9C); also *CPS1* showed a dose dependent decrease (Supplementary Fig. 9C) leading to compromised ammonia metabolism (Supplementary Fig. 10). Using the IM [4], we also observed a discrepancy between the predicted and measured ammonia in the dose dependent study (Fig. 1F).

To find an explanation for this discrepancy, we performed time-resolved gene array analysis of mouse liver tissue after CCl_4 intoxication (Fig. 2A). Fuzzy clustering identified seven gene clusters, which reflected time dependent gene expression alterations [6]. Clusters 4 and 6 contained genes whose expression was transiently repressed at early time points after CCl_4 intoxication (Fig. 2B). Further bioinformatics analyses revealed an over representation of nitrogen/ammonia metabolism KEGG and Gene ontology terms of genes in cluster 4 (Fig. 2C, D). Genes relevant for ammonia metabolism were further studied by qRT-PCR, immunostaining and activity assays. GS is the key enzyme for ammonia detoxification in the pericentral compartment. RNA levels of GS started to decrease as early as 6 h after CCl_4 injection, it was at its lowest between days 1 and 4, before finally recovering to initial levels between days 6 and 30 (Fig. 2E). A similar time-dependent curve was obtained for GS activity although the decrease occurred slightly later than that of RNA with very low levels between days 2 and 4 (Fig. 2E). The pattern and intensity of GS immunostaining was found to be comparable to GS activity (Fig. 2F). In addition, ornithine aminotransferase (OAT), an enzyme exclusively localized in GS positive pericentral hepatocytes that provides additional glutamate for fixing ammonia [7], decreased to almost undetectable levels with a delayed recovery (Supplementary Fig. 3A). The key enzymes of the periportal compartment, CPS1, ASS1, ASL and arginase1 were similarly analyzed in the same tissue (Supplementary Figs. 3B and