Partial deletion of argininosuccinate synthase protects from pyrazole plus lipopolysaccharide-induced liver injury by decreasing nitrosative stress

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Lu Y, Leung TM, Ward SC, Nieto N. Partial deletion of argininosuccinate synthase protects from pyrazole plus lipopolysaccharide-induced liver injury by decreasing nitrosative stress. Am J Physiol Gastrointest Liver Physiol 302: G287–G295, 2012. First published November 3, 2011; doi:10.1152/ajpgi.00375.2011.—Argininosuccinate synthase (ASS) is the rate-limiting enzyme in the urea cycle. Along with nitric oxide synthase (NOS)-2, ASS endows cells with the t-citrulline/nitric oxide (NO) salvage pathway to continually supply t-arginine from t-citrulline for sustained NO· generation. Because of the relevant role of NOS in liver injury, we hypothesized that downregulation of ASS could decrease the availability of intracellular substrate for NO· synthesis by NOS-2 and, hence, decrease liver damage. Previous work demonstrated that pyrazole plus LPS caused significant liver injury involving NO· generation and formation of 3-nitrotyrosine protein adducts; thus, wild-type (WT) and Ass−/− mice (Ass−/− mice are lethal) were treated with pyrazole plus LPS, and markers of nitrosative stress, as well as liver injury, were analyzed. Partial ablation of Ass protected from pyrazole plus LPS-induced liver injury by decreasing nitrosative stress and hepatic and circulating TNFα. Moreover, apoptosis was prevented, since pyrazole plus LPS-treated Ass−/− mice showed decreased phosphorylation of JNK; increased MAPK phosphatase-1, which is known to deactivate JNK signaling; and lower cleaved caspase-3 than treated WT mice. and this was accompanied by less TdT-mediated dUTP nick end labeling-positive staining. Lastly, hepatic neutrophil accumulation was almost absent in pyrazole plus LPS-treated Ass−/− compared with WT mice. Partial Ass ablation prevents pyrazole plus LPS-mediated liver injury by reducing nitrosative stress, TNFα, apoptosis, and neutrophil infiltration.

urea cycle; t-citrulline/nitric oxide cycle; nitric oxide synthase-2

THE UREA CYCLE IS A METABOLIC pathway in which ammonia produced during protein catabolism is converted to urea in the liver. The cycle involves five reactions occurring between the mitochondria and the cytosol: 1) 2ATP + HCO₃⁻ + NH₄⁺ → carbamoyl phosphate + 2ADP + Pᵢ (carbamoyl phosphate synthase-1); 2) carbamoyl phosphate + ornithine → citrulline + Pᵢ (ornithine transcarbamylase); 3) t-citrulline + aspartate + ATP → argininosuccinate + AMP + PP₁ (argininosuccinate synthase (ASS)); 4) argininosuccinate → arginine + fumarate (arginino- succinate lyase); and 5) arginine + H₂O → ornithine + urea (arginase). ASS catalyzes the reversible ATP-dependent condensation of t-citrulline and aspartate to form argininosuccinate, the immediate precursor of t-arginine (14). Since ASS is the enzyme with the lowest Vₘₐₓ, it is considered the rate-limiting enzyme in the urea cycle (14). Ass deficiency in humans, a rare genetic disorder associated with high mortality, results in citrullinemia (2). Hyperammonemia and reduction in urea synthesis occur in alcoholic and cirrhotic patients, many of whom have low ASS activity (36). Ass−/− mice die within 24 h due to hyperammonemia, hepatic encephalopathy, and liver failure (43).

ASS and argininosuccinate lyase are usually considered in the context of their contribution to the urea cycle in the liver; yet, in conjunction with nitric oxide synthase-2 (NOS2), they endow cells with a salvage pathway, the t-citrulline/nitric oxide (NO) cycle, that continually generates t-arginine from t-citrulline for sustained NO· generation (6, 14). ASS overexpression leads to enhanced NO· generation (41, 42). Type I citrullinemic patients (due to ASS deficiency) show decreased serum nitrates plus nitrites, and NO· synthesis remains low, despite dietary supplementation of arginine (24). The role of the t-citrulline/NO· cycle in the liver, the potential role of ASS as an enzymatic “switch” to provide substrate for NOS2-induced activity, and the subsequent excess of NO· biosynthesis during liver injury remain to be defined.

NOS2 mediates a variety of pathological conditions in the liver (10, 37), since it produces NO·-derived prooxidants (22, 37). NOS2-mediated NO· generation has been implicated in epithelial cell injury, apoptosis, host immune defense, and perpetuation of the inflammatory response (10). While the NOS2 gene lies quiescent under physiological conditions, certain factors, such as alcohol and LPS, can initiate and sustain its activation (41).

Conduction of NOS2 and ASS has been demonstrated in vivo in various tissues and cells treated with LPS (7, 12, 13, 25, 28). In the liver, hepatocytes can be stimulated to generate large amounts of NO·, along with urea, in response to sepsis or LPS (29). This raises the following question: Could ASS contribute to liver injury by activating NOS2, with the resulting regulation of NO· synthesis? Thus, ASS could have a rate-limiting role for high-output NO· synthesis via NOS2.

Virtually nothing is known about the mechanism whereby modulation of ASS expression and the activity of the t-arginine recycling pathway may affect NO· generation and liver injury. Thus we hypothesized that downregulation of ASS could decrease the availability of intracellular substrate for NO· synthesis by NOS2 and, hence, decrease liver injury. We previously demonstrated that pyrazole plus LPS treatment caused significant liver injury involving NO· generation and formation of 3-nitrotyrosine (3-NT) protein adducts (20). Moreover, administration of DW1400, a NOS2 inhibitor, decreased liver injury induced by pyrazole plus LPS (38). In the present study, we show that partial Ass deficiency lowers nitrosative stress and decreases pyrazole plus LPS-mediated liver injury by reducing nitrosative stress, TNFα, apoptosis, and neutrophil infiltration.

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MATERIALS AND METHODS

General methodology. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using kits from Pointe Scientific (Canton, MI). Serum and hepatic TNFα levels were measured using a kit from Invitrogen (Carlsbad, CA). Liver nitrates plus nitrates were measured using the Griess reagent (Cayman Chemical, Ann Arbor, MI).

Mice. Ass<sup>+/−</sup> (B6;129S7-Ass<sup>tm1Bay</sup>/J) mice, TNFα receptor-1 knockout (B6.129-Tnfrsf1a<sup>tm1Bov</sup>; Tnf<sup>fl/fl</sup>) mice, and their wild-type (WT) littermates were purchased from Jackson Laboratory (Bar Harbor, ME). Mice homozygous for the Ass<sup>tm1Bov</sup> mutation stop gaining weight a few hours after birth and usually die within 24 h due to hyperammonemia (32). Heterozygous mice are healthy and develop normally; however, plasma t-citrulline levels are increased twofold in heterozygous mice over their WT siblings (32). All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Protocols were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee.

Model of pyrazole plus LPS-mediated liver injury. Mice were injected with pyrazole (Sigma, St. Louis, MO; 150 mg/kg body wt ip) once per day for 2 days (20). Controls were injected with 0.9% NaCl. After an overnight fast, LPS (Sigma, serotype 055:B5), with a biological activity ≥500,000 endotoxin units/mg measured by the Limulus amebocyte lysate assay, was injected (4 mg/kg body wt ip). Controls were injected with 0.9% NaCl. All mice were killed 24 h after the LPS or NaCl injections. Blood was collected by orbital venous plexus puncture. Liver nitrites plus nitrates were measured using the Griess reagent (37), and the enhanced chemiluminescence reaction was developed and quantified (38). Liver nitrites plus nitrates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Pathology. The left liver lobe from all mice was collected and fixed in 10% neutral-buffered formalin and processed into paraffin sections for hematoxylin-eosin or immunohistochemical (IHC) staining. The hematoxylin-eosin-stained sections were evaluated by a liver pathologist blinded to the experimental conditions. Ten ×100 fields were examined for necroinflammatory activity, which was scored as follows: 0 for none, 1 for <2, 2 for 2–4, 3 for 5–10, and 4 for >10 foci per ×100 field. The density of the necroinflammatory activity was also calculated per square millimeter over the ten 100 field. The density of the necroinflammatory activity was calculated per square millimeter over the ten 100 field. The density of the necroinflammatory activity was calculated per square millimeter over the ten 100 field. The density of the necroinflammatory activity was calculated per square millimeter over the ten 100 field. The density of the necroinflammatory activity was calculated per square millimeter over the ten 100 field. The density of the necroinflammatory activity was calculated per square millimeter over the ten 100 field. The density of the necroinflammatory activity was calculated per square millimeter over the ten 100 field.

Western blot analysis. The Abs for ASS, actin, JNK (54 and 46 kDa), phosphorylated JNK (54 and 46 kDa), MAPK phosphatase-1, cellular caspase-8-like inhibitory protein long and short isoforms (cFLIP-L and cFLIP-S), cleaved caspase-3, and arginine residues were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Ab for 3-NT was obtained from Chemicon, and the Abs for TNFα and catalase were obtained from Chemicon (Temecula, CA). The enhanced chemiluminescence reaction was developed and quantified using a scanner (model Las4000, Fujifilm, Stamford, CT), and the intensity of the signal was quantified using ImageJ software (National Institutes of Health). All samples from the same experiment were run on the same gel and transferred onto the same nitrocellulose membrane. In all experiments, actin was the loading control.

Results

Partial Ass ablation protects from pyrazole plus LPS-induced liver injury. We previously reported that pyrazole or LPS alone did not cause significant liver injury (20); however, pyrazole plus LPS, at the same doses used in this study, induced major liver injury in mice under two different genetic backgrounds (C57BL/6 and 129sv) (20). To examine whether ASS contributes to the pyrazole plus LPS-induced liver damage, Ass<sup>−/−</sup> mice were used, since Ass<sup>−/−</sup> mice are lethal. ASS expression was 80% lower in Ass<sup>−/−</sup> than WT mice and was further decreased by 60% in WT mice treated with pyrazole plus LPS (Fig. 1A); liver arginine, quantified by the presence of t-arginine residues, was lower in pyrazole plus LPS-treated Ass<sup>−/−</sup> mice than WT mice, suggesting less supply of substrate (t-arginine) for the NO<sub>2</sub>S2 reaction to generate NO· (Fig. 1B). Serum ALT and AST activities increased four- and sevenfold, respectively, in pyrazole plus LPS-treated WT mice but were only slightly augmented in Ass<sup>−/−</sup> mice (Fig. 1, C and D). Hematoxylin-eosin staining and the pathology scores for necrosis and inflammation demonstrated that pericentral necrosis and inflammation were excessive in pyrazole plus LPS-injected WT mice and minimal in Ass<sup>−/−</sup> mice (Fig. 1, E–G). These results suggest that partial Ass ablation protects from pyrazole plus LPS-induced liver injury.

Partial Ass deletion prevents pyrazole plus LPS-induced nitrosative stress. Cytochrome P-450 2E1- and 2A5-related oxidative stress played a role in the model of pyrazole plus LPS-induced liver injury previously described (20). However, no significant changes in cytochrome P-450 2E1 and 2A5 along with catalase were observed between WT and Ass<sup>−/−</sup> mice after pyrazole plus LPS treatment (data not shown), ruling out different pyrazole metabolism or an additional contribution of these enzymes to the effects observed by the pyrazole plus LPS injection. ASS, an enzyme shared by the urea and L-citrulline/NO· cycles, could condition NO· availability via arginine supply and NO<sub>2</sub>S2 activity. IHC analysis showed NOS2 induction mainly in zone 3 (pericentral area) in pyrazole plus LPS-injected WT mice; however, the NOS2-positive staining was lower in treated Ass<sup>−/−</sup> mice (Fig. 2, A and B). This indicated that ASS is required for NOS2 induction by pyrazole plus LPS.

Consistently, a significant increase in NO· production, measured as the hepatic concentration of nitrates plus nitrates, soluble and stable metabolites of NO·, was observed in WT mice following pyrazole plus LPS treatment; nevertheless, NO· production was lower in Ass<sup>−/−</sup> mice (Fig. 2C). NO· could lead to posttranslational modifications in protein tyrosines to generate 3-NT residues (9, 15). Western blot analysis showed that pyrazole plus LPS treatment increased 3-NT protein adducts only in WT mice (Fig. 2D). Moreover, 3-NT IHC revealed positive staining around pericentral areas, which paralleled results of NOS2 staining (Fig. 2, E and F). These results suggest that greater nitrosative stress was induced in WT than Ass<sup>−/−</sup> mice, and these findings could explain less liver injury by pyrazole plus LPS treatment in Ass<sup>−/−</sup> than WT mice.

Pyrazole plus LPS treatment upregulates TNFα in WT compared with Ass<sup>−/−</sup> mice. The pyrazole plus LPS injection model of liver injury significantly increased TNFα production.
In addition, NOS2 is a critical mediator of Bacillus Calmette-Guérin plus LPS-induced liver injury, contributing to secretion of proinflammatory cytokines such as TNFα (11, 31).

To examine the potential role of TNFα in liver injury induced by pyrazole plus LPS injection, liver TNFα-positive staining and concentration, along with serum TNFα levels, were measured in WT and Ass+/− mice. As shown in Fig. 3, after pyrazole plus LPS injection, more TNFα-positive sinusoidal staining was observed in WT than Ass+/− mice (Fig. 3, A and B). Hepatic TNFα levels were twofold higher in cotreated WT than Ass+/− mice (Fig. 3D). Serum TNFα levels increased 22-fold in pyrazole plus LPS-injected WT mice and 10-fold in Ass+/− mice (Fig. 3C).

To further confirm the role of TNFα in this model of liver injury, Tnfr1−/− mice and their WT littermates were injected with pyrazole plus LPS. Liver injury was significantly enhanced in WT, but not Tnfr1−/−, mice, as demonstrated by serum ALT and AST activities (Fig. 3, E and F). These results
suggest that TNFα plays an essential role in liver injury induced by pyrazole plus LPS.

Pyrazole plus LPS injection increases phosphorylation of JNK and induces apoptosis more in WT than Ass+/− mice. The role of TNFα in cell survival and cell death is determined by the balance between NF-κB and JNK signaling (3). NF-κB promotes survival, whereas JNK enhances cell death (3). TNFα-mediated JNK activation accelerates the turnover of the NF-κB-induced antiapoptotic protein cFLIP (3). As shown in Fig. 4A, after pyrazole plus LPS treatment, JNK phosphorylation was observed in the p54, but not p46, isoform of JNK in WT and Ass+/− mice, although it was much greater in WT mice. In contrast, MAPK phosphatase-1, which can deactivate JNK signaling (3), was induced in pyrazole plus LPS-injected mice.
Ass\(^{+/−}\) compared with WT mice (Fig. 4A). Cleaved caspase-3 was significantly higher in pyrazole plus LPS-injected WT than Ass\(^{+/−}\) mice (Fig. 4A). Next, DNA fragmentation was measured by the TUNEL assay. Positive TUNEL staining occurred in pyrazole plus LPS-treated WT mice, but it was notably reduced in Ass\(^{+/−}\) mice and completely absent in untreated mice (Fig. 4B). The number of TUNEL-positive nuclei was quantified, and the TUNEL index was calculated and found to be fivefold higher in pyrazole plus LPS-treated WT than Ass\(^{+/−}\) mice (Fig. 4C).

Neutrophil infiltration is increased in pyrazole plus LPS-treated WT compared with Ass\(^{+/−}\) mice. Because inflammatory cells can adhere to the sinusoidal wall and accumulate damaging hepatocytes mainly via generation of reactive oxygen...
species and LPS induces neutrophil infiltration and, hence, drives liver injury (19), we evaluated the presence of hepatic neutrophils in this model. As shown in Fig. 5A, neutrophil infiltration was absent in control WT and Ass−/− mice; however, there was significant neutrophil accumulation in the liver parenchyma in pyrazole plus LPS-treated WT compared with Ass−/− mice, in which neutrophils were confined to the sinusoids. The presence of neutrophils was quantified by positive

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**Fig. 4.** Pyrazole plus LPS injection increases phosphorylation of JNK and induces apoptosis in WT compared with Ass−/− mice. Western blot analysis showed higher phosphorylated JNK (pJNK, p54 isoform) and cleaved caspase-3, along with lower MAPK phosphatase-1 (MKP1), protein expression in pyrazole plus LPS-injected WT than Ass−/− mice. Similar changes were observed for cellular caspase-8-like inhibitory protein long and short isoforms (cFLIP-L and cFLIP-S) between pyrazole plus LPS WT and Ass−/− mice. 

A: blots and quantification of the intensity of each signal corrected by actin signal intensity. B: DNA fragmentation determined by TdT-mediated dUTP nick end labeling (TUNEL) shows positively stained nuclei in livers from WT mice injected with pyrazole plus LPS, while staining in Ass−/− mice was almost negative. C: TUNEL staining quantified by morphometry analysis. Values are means ± SE; n = 6. ***P < 0.01, ***P < 0.001 vs. control. –P < 0.001 vs. WT.
naphthol AS-D chloroacetate esterase staining and showed an eightfold increase in WT compared with Ass<sup>+/−</sup> mice (Fig. 5B). These results suggest that partial Ass deletion plays a role in preventing inflammation and, hence, contributes to avert liver injury.

**DISCUSSION**

Since nitrosative stress plays a central role in liver injury and ASS is a critical enzyme from the urea and the l-citrulline/NO· cycles, which could have a rate-limiting role in high-output NO· synthesis via NOS2, we hypothesized that downregulation of ASS could decrease the availability of arginine, an intracellular substrate for NO· synthesis by NOS2, and, therefore, decrease liver injury.

To assess the biological relevance of ASS for NO· synthesis in vivo and its potential role in protecting from liver injury, WT and Ass<sup>+/−</sup> mice were subjected to a model of liver injury based on pyrazole plus LPS injection, where nitrosative stress is a critical trigger (20, 38, 40). Although hepatic ASS content decreased after pyrazole plus LPS injection, this in vivo model of liver injury showed less nitrosative stress, lower intrahepatic and circulating TNFα, and decreased apoptosis and hepatic neutrophil infiltration in Ass<sup>+/−</sup> compared with WT mice. Thus we report that a urea and l-citrulline/NO· cycle enzyme could promote nitrosative stress and pyrazole plus LPS-mediated liver injury.

Regarding the mechanism whereby ASS causes injury in this scenario, a key and logical conjecture was its possible role in regulating NO· generation via NOS2 activation in hepatocytes. Simultaneous induction of NOS2 and ASS has been demonstrated in vivo in various tissues and cells treated with LPS (7, 12, 13, 25, 28). ASS overexpression leads to an enhanced ability for NO· production (41). Serum levels of nitrates plus nitrites are 40% lower in type I citrullinemic patients than in healthy individuals (24). Once NOS2 is highly induced, as it is during liver injury, cellular NO· overproduction is determined not only by NOS2 activity but also by the intracellular concentration of l-arginine, which is the only physiological nitrogen donor for NO· production (23), and by the availability of cofactors for the NOS2 reaction. An active urea cycle provides a ready source of l-arginine for high-output NO· synthesis in the liver. Indeed, Ass deficiency in mice lowered l-arginine levels, reflected by decreased arginine residues in the liver upon the onset of liver injury; furthermore, NOS2 induction was lower in Ass<sup>+/−</sup> than WT mice. As a consequence, hepatic nitrates plus nitrites, stable and soluble metabolites of NO·, were higher in pyrazole plus LPS-treated WT than Ass<sup>+/−</sup> mice. Peroxynitrite, generated by the rapid reaction of NO· with superoxide radical, nitrates free and protein-associated tyrosine residues (16). 3-NT protein adducts, the footprint of nitrosative stress, were increased after pyrazole plus LPS treatment in WT compared with Ass<sup>+/−</sup> mice, suggesting that ASS activity contributes to nitration reactions resulting in enhanced liver damage in WT mice.

NOS2 is expressed in hepatocytes and in nonparenchymal cells; however, ASS is only expressed in hepatocytes, where active urea and l-citrulline/NO· cycles occur. Therefore, it is likely that ASS promotes liver injury via hepatocyte NOS2. In addition to NOS2, hepatocytes also express NOS3, which has been described to play a protective role in alcohol-induced
liver injury (22, 26). It is conceivable that NOS3 activity could be lower in Ass<sup>+/−</sup> than WT mice, under conditions that promote liver injury, such as alcoholic liver disease (unpublished observations); however, despite a possible decrease in NOS3 activity, pyrazole plus LPS treatment still induced minor liver injury in Ass<sup>+/−</sup> compared with WT mice, perhaps because the effects of NO· from NOS3 may have been lost during endotoxemia (5, 8).

Pyrazole plus LPS-induced liver injury leads to TNFα induction (38, 40). In this study, pyrazole plus LPS increased serum AST and ALT in WT, but not Tnfα<sup>−/−</sup>, mice, validating the role of TNFα in the pyrazole plus LPS model of liver damage. Indeed, pyrazole plus LPS treatment increased hepatic and circulating TNFα in WT compared with Ass<sup>+/−</sup> mice. Since TNFα-positive staining mainly localized to the sinusoids, it is likely that neutrophils, Kupffer cells, and hepatic stellate cells could be the main source of TNFα (4, 33). How ASS regulates TNFα production remains unclear. It has been reported that LPS may cause the release of ASS into the bloodstream and that this circulating ASS could decrease TNFα (34). This may not be the case in our model, since Ass<sup>+/−</sup> mice may presumably release less ASS, at least no more than WT mice, while they have lower serum levels of TNFα. It has been reported that NOS2 is a critical mediator of Bacillus Calmette-Guérin plus LPS-induced increases in circulating proinflammatory cytokines such as TNFα and liver injury (11, 31); thus it is possible that the lower NOS2 induction in injured Ass<sup>+/−</sup> mice could have conditioned the increase in TNFα. Yet this possibility needs further evaluation.

Oxidative and nitrosative damage to the mitochondria can alter mitochondrial membrane potential and permeability transition (1, 35) and trigger mitochondrial swelling, cristae fracture, cytochrome c release, further oxidative stress, and, ultimately, apoptosis via activation of the caspase signaling cascade (17, 18, 27, 39). In addition, TNFα participates in cell survival and cell death pathways (3). The balance between NF-κB and JNK determines the biological outcome of TNFα, and while NF-κB promotes survival, JNK enhances cell death (3). NF-κB induces the expression of cFLIP, an inhibitor of caspase-8, whereas JNK promotes cFLIP-L ubiquitination and proteosomal degradation (3). After pyrazole plus LPS treatment, JNK phosphorylation (p54) increased, while cFLIP-L decreased, more in WT than Ass<sup>+/−</sup> mice. Similarly, cleaved caspase-3 and positive TUNEL staining increased in treated WT compared with Ass<sup>+/−</sup> mice. These results suggest that, under pyrazole plus LPS treatment, partial ablation of Ass lowers secreted TNFα, which in turn downregulates JNK signaling and apoptosis.

Under normal conditions, apoptotic bodies are rapidly phagocytosed; however, when there is imbalance between the rate of apoptosis and phagocytosis, necrosis and inflammation occur (30). Neutrophils are the first line of protection and are recruited by sensing chemical gradients mostly triggered by injured cells. Neutrophils migrate toward apoptotic cells in an effort to clear them, a process that can also be noxious for neighboring healthy liver cells (19, 21). LPS treatment caused sequestration of neutrophils in the hepatic sinusoids, which in itself did not result in significant liver injury; however, when LPS was administered along with a hepatotoxin such as galactosamine, a significant number of neutrophils infiltrated the hepatic parenchyma and caused gross tissue destruction (19, 21). Similarly, in the model of pyrazole plus LPS, LPS alone did not induce liver injury, but LPS plus pyrazole induced hepatocyte apoptosis in a time-dependent manner and at last caused severe liver injury (18). In this study, after pyrazole plus LPS treatment, hepatic apoptosis and neutrophil infiltration into the liver parenchyma were observed mostly in WT mice, but in Ass<sup>+/−</sup> mice, less hepatic apoptosis was observed and neutrophils were mainly confined to the sinusoids; thus it is conceivable that the ongoing apoptosis may have triggered infiltration of neutrophils into the liver parenchyma, thus amplifying liver damage.

Elucidation of the factors and pathways that condition NOS2 activity could help in the design of therapies to limit pathophysiological NO· production, as in advanced liver disease. The approach used in this study helped define the contribution of ASS to liver injury. Thus we conclude by suggesting that ASS plays an important role as an enzymatic switch for governing NO· production by NOS2 during liver injury, perhaps by supplying the substrate. It remains an open question whether generation of reactive nitrogen species upon the onset of liver injury leads to relevant posttranslational modifications in proteins, possibly within metabolic pathways such as the urea and l-citrulline/NO· cycles, with the subsequent loss or gain of function.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.L. and N.N. are responsible for conception and design of the research; Y.L., T.M.L., and N.N. prepared the figures; Y.L. and N.N. drafted the manuscript; Y.L., T.M.L., and N.N. edited and revised the manuscript; N.N. approved the final version of the manuscript.

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