

Effect of L-NAT on hepatic ischemia-reperfusion injury via PI3K-Akt signaling pathway

Jianxin Wang^{1,*}, Shuna Yu^{1,*}, Huiting Li¹, Hongxin Jiang³, Peilun Xiao¹, Yitong Pan¹, Zhe Wang¹, Jie Zheng⁴, Jiying Jiang^{1,*}

¹Departments of Anatomy, Weifang Medical University, Weifang, Shandong 261053, China

²Departments of Histology and Embryology, Weifang Medical University, Weifang, Shandong 261053, China

³ Morphology Lab. Of, Weifang Medical University, Weifang, Shandong 261053, China

⁴Departments of Pathology, Weifang Medical University, Weifang, Shandong 261053, China

*Correspondence should be addressed to Jiying Jiang: 13059084277@163.com

These authors contributed equally to this work.

Abstract

Objective: N-acetyl-L-tryptophan (L-NAT) is a NK1 (Neurokinin 1) receptor antagonist, SP (substance P) and its specificity NK-1R binding involves the occurrence of ischemia, reperfusion injury in organs such as heart, brain and liver. PI3K-Akt signal is an important survival signal transduction pathway in vivo and plays an important role in ischemia-reperfusion injury. In this study, animal models of liver warm ischemia-reperfusion injury were used to investigate whether L-NAT can protect hepatic ischemia-reperfusion injury by activating PI3K-Akt pathway and provide experimental basis for the application of L-NAT in hepatic diseases. **Methods:** Forty-eight adult SD rats were randomly divided into 4 groups: Sham group, HIRI group, L-NAT pretreatment group (L-NAT+HIRI group), LY294002 pretreatment (LY294002+L-NAT+HIRI) group. In the Sham group, only the hepatic hilum was isolated and no other operations were performed; In the HIRI group, a partial liver warm ischemia-reperfusion injury model was established by clamping the branches of the middle and left hepatic pedicles with a vascular clamp for 45 min and reperfusion for 6 h; In the L-NAT preconditioning group, L-NAT 10 mg/kg was intra-peritoneally injected 30 min before ischemia, and the other operations were the same as those in the HIRI group; LY294002 1.5 mg/kg was injected into the LY294002+L-NAT+HIRI group 1 h before ischemia in the tail vein, and the other operations were the same as L-NAT+HIRI group. After 6 h, obtain liver tissue and blood. The liver tissues were routinely embedded in paraffin, sectioned, stained with HE, and photographed; Colorimetric assay was used to detect the activity of alanine aminotransferase (ALT) in serum. **Results:** (1) HE staining: The hepatocytes in hepatic ischemia reperfusion injury were swollen and the structure was disordered. After L-NAT intervention, the structure of hepatic lobule was basically complete and the cell swelling disappeared; LY294002 intervention could antagonize the protective effect of L-NAT. (2) Hepatocyte function tests showed that serum ALT levels in HIRI group were higher than those in Sham group (284.28 ± 9.81 U/mgprot vs 63.83 ± 9.10 U/mgprot) ($P < 0.01$); ALT activity after L-NAT intervention decreased (124.90 ± 10.62 U/mgprot vs 284.28 ± 9.81 U/mgprot) ($P < 0.01$); LY294002 reversed the above-mentioned effect of L-NAT (230.55 ± 12.98 U/mgprot vs 124.90 ± 10.62 U/mgprot) ($P < 0.01$). (3) Western blotting showed that the expression of p-AktSer473 protein was consistent with that of Akt mRNA. **Conclusion:** (1) L-NAT has protective effect on hepatic ischemia-reperfusion injury. (2) The protective effect of L-NAT is achieved by activating the PI3K-Akt signal transduction pathway.

Keywords

Hepatic ischemia-reperfusion injury; Apoptosis; L-NAT; PI3K-Akt.

1. Introduction

Liver ischemia-reperfusion injury often occurs during liver transplantation, tumor resection, liver trauma, hemorrhagic shock, etc. Liver ischemia-reperfusion injury not only damages the liver, but also separate organs such as heart, brain, lung and kidney. It also causes certain damage, which is not conducive to the recovery of liver function in patients, and even causes liver failure or multiple organ failure. Hepatic ischemia-reperfusion injury has become a key factor affecting the recovery of patients and determining the success or failure of surgery. At present, the prevention and treatment measures for hepatic ischemia-reperfusion injury mainly include drug pretreatment, ischemic preconditioning, ischemic postconditioning, and mild hypothermia treatment. Among them, the most widely studied are drug pretreatment [1] and ischemic preconditioning. [2-3]. Although the protective effect of ischemic preconditioning has been verified, this method requires increased surgical procedures and hemorrhage, which is a traumatic and invasive procedure. To be truly applied clinically, there is still considerable concern among the patient's family and clinicians. The clinical application of ischemic preconditioning is difficult to promote. Drug pretreatment is the use of active drugs to initiate endogenous protection mechanisms to increase the tolerance of tissues and cells to ischemia-reperfusion injury, and reduce the incidence and degree of ischemia-reperfusion injury [4].

Numerous studies have shown that SP-mediated neurogenic inflammatory responses play a role in inflammation, tumorigenesis and ischemia-reperfusion injury. SP-specific antibody NK-1R gene knockdown or the use of NK-1R antagonists can alleviate the above lesions[5-9]. The P13K-Akt signal transduction pathway is an important signaling pathway in vivo, and is mainly involved in various life activities such as cell proliferation, differentiation, survival, apoptosis and malignant transformation. Previous studies have shown that both drug preconditioning and ischemic preconditioning can activate the P13K/Akt signaling pathway, thereby reducing ischemia-reperfusion injury in kidney, heart, liver, brain and other organs [10-14].

Previous studies in our laboratory have also confirmed that L-NAT can attenuate neuronal apoptosis and increase cell viability caused by cerebral ischemia and hypoxia injury [15]. Previous studies have shown that the expression levels of SP and NK-1R in ischemia, reperfusion injury of liver, brain, heart and other organs have increased significantly [16-18]. Based on the above findings, it can be hypothesized that blocking the effects of SP and NK-1R with an NK-1R antagonist (eg, LY294002) may attenuate ischemia-reperfusion injury.

2. Materials and methods

2.1 Animals and Treatments

Male SD rats, weighing between 200g and 220g, were purchased from Jinan Dion Shopping Jinan Pengyue Experimental Animal Center. The animals were randomly divided into two groups: sham group (n=10) and HIRI group (n=10). The HIRI model was prepared with reference to the previous literature[12]. The Animal Ethics Committee of the University approved all working protocols.

2.2 Immunofluorescence technique

The cells were treated as above and fixed in 4% paraformaldehyde for 15min, incubated in 0.3% Triton X-100-PBS for 10 min at room temperature and blocked with 5% goat serum for 30 min at 37°C. The liver tissue of each group was fixed with 4% paraformaldehyde for 48 hours, embedded in paraffin, sliced (10 μm thick). Immunofluorescence staining of TLR4 (1:200; Bioss, Beijing, bs-0065R) and NF-κB(1:200; Bioss, Beijing, bs-0064R) was performed by routine methods. The reaction was followed by FITC-conjugated secondary antibodies (1:150) and covered with Mounting Medium with DAPI.

2.3 Western Blot Analysis

The total protein was abstracted from the BRL cells, cells were washed twice with ice-cold PBS (pH7.4) and using RIPA buffer (Solarbio, Beijing, China). Proteins were subjected to SDS-PAGE with a 10% running gel, and then transferred to a PVDF membrane. Incubated with the following

antibodies: anti-NF- κ B (1:500, Bioss, Beijing, bs-0465R); anti-TLR-4 (1:500, Bioss, Beijing, bs-1021R); anti-Rip2 (1:500, Santa); anti-GAPDH (1:2000, proteintech, UBS, 10494-1-AP), respectively. The details for the analysis have been described in previous literature [12]. Western blot images were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA). GAPDH was used as the Internal reference protein.

3. Result

3.1 Effect of L-NAT on hepatocyte morphology after ischemia-reperfusion

The liver tissue samples of each group were found by HE staining. The liver tissue structure of Sham group was obvious, the level was clear, the cells were arranged regularly, the cell structure was normal, no damage and loss, nucleoli solidification, chromatin uniformity; HIRI group appeared between liver tissues Edema, disordered cells, swelling and rupture of hepatocytes; edema between liver tissues in L-NAT+HIRI group improved, only a few cells were disordered, and the degree of cell damage was reduced; LY294002+L-NAT+HIRI group could reverse L-NAT The protective function, staining, and significant edema between liver tissues were not significantly different from those in the HIRI group (Fig. 1).

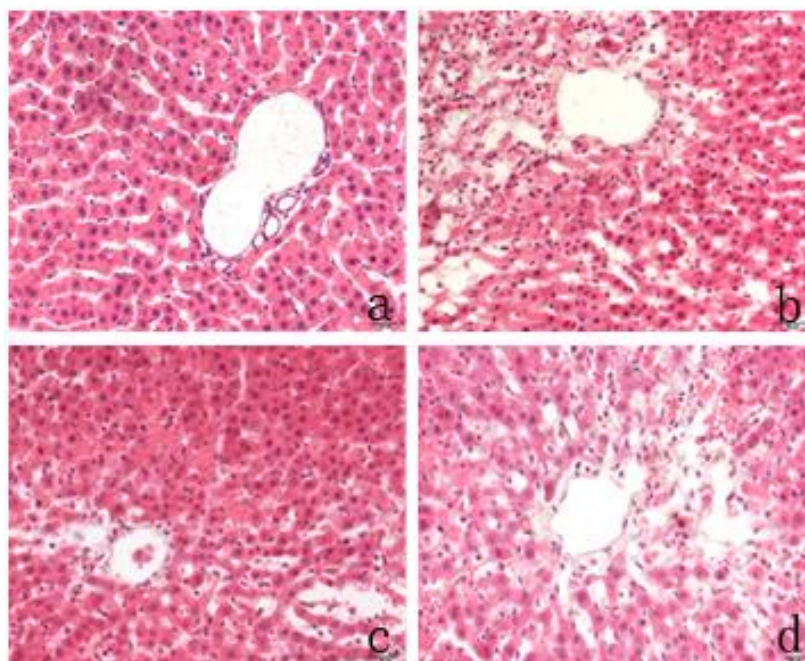


Figure 1 L-NAT can alleviate the morphological changes of hepatocytes caused by I/R injury(400 \times)

Note: vs Sham group * $P < 0.01$, vs HIRI group # $P < 0.01$, vs L-NAT+HIRI group & $P < 0.01$

3.2 Effect of L-NAT on liver function after ischemia-reperfusion

Hepatocyte function tests showed an increase in serum ALT levels in the HIRI group compared with the Sham group (284.28 ± 9.81 U/mg prot vs 63.83 ± 9.10 U/mg prot) ($P < 0.01$); ALT activity decreased after L-NAT intervention (124.90 ± 10.62 U/mgprot vs 284.28 ± 9.81 U/mgprot) ($P < 0.01$); LY294002 reversed the above effects of L-NAT (230.55 ± 12.98 U/mgprot vs 124.90 ± 10.62 U/mgprot) ($P < 0.01$) (Table 1, Figure 2).

3.3 Effect of L-NAT on the expression of p-AktSer473 in liver tissue after ischemia-reperfusion

Western blotting showed that p-AktSer473 protein showed a specific band around 56kDa. Compared with Sham group, the expression of p-AktSer473 protein in HIRI group was increased ($P < 0.01$), which was statistically significant. Compared with HIRI group, The expression of p-AktSer473 protein in NAT+HIRI group was significantly increased ($P < 0.01$). The expression of p-AktSer473 protein in LY294002+L-NAT+HIRI group was lower than that in L-NAT+HIRI group ($P < 0.01$). Statistically significant (Figure 3).

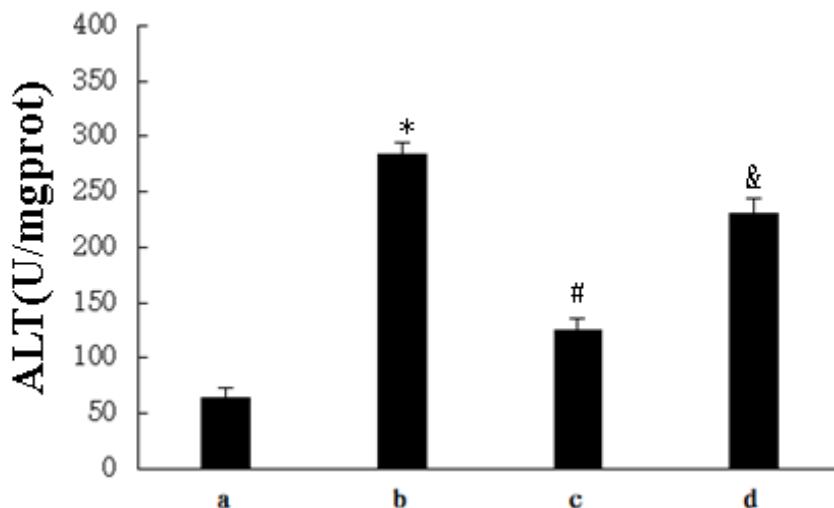


Figure 2 serum ALT activity of each group (n=6)
 a:Sham b:HIRI c:L-NAT+HIRI d:LY294002+L-NAT+HIRI

Table 1 Serum ALT activity of each group (±S, n=6) (U/mgprot)

组别	ALT
Sham	63.83±9.10
HIRI	284.28±9.81*
L-NAT+HIRI	124.90±10.62#
L-NAT+HIRI+LY294002	230.55±12.98&

Note: vs Sham group *P<0.01 5, vs HIRI group #P<0.01, vs L-NAT+HIRI group &P<0.01

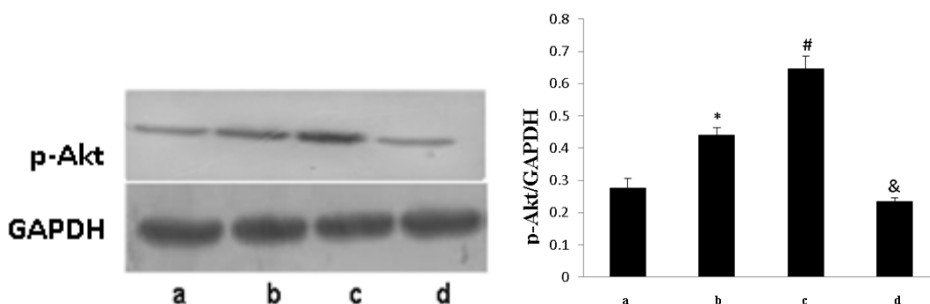


Figure 3 p-AktSer473 protein expression in liver tissue of each group (n=6)
 a:Sham b:HIRI c:L-NAT+HIRI d:LY294002+L-NAT+HIRI

4. Discussion

Ueno T et al. used immunohistochemistry to localize substance P in normal human liver biopsy. It was found that SP-positive nerve fibers were mainly distributed around the hepatic sinus and hepatocytes and around the hilar and bile ducts. The distal ends were mostly located in the sinusoids. Vascular endothelial cells and fibroblasts, suggesting that SP is involved in hepatic lobular hemodynamic regulation [19]. Lee FY et al found that the level of SP in the blood of patients with cirrhosis was significantly higher than that of the healthy control group; while the level of SP in patients with decompensated cirrhosis was higher than that of patients with compensated cirrhosis, accompanied by higher venous pressure and lower Vascular resistance [20]. El-Raziky et al found that children with chronic liver disease had elevated levels of SP in the blood and upregulated aldosterone and renin activity, and the correlation indicates that SP has a potential effect on the prognostic index of the disease [21]. Through a series of discoveries, Bang R's laboratory has

expressed a variety of NK-1R cells in the liver; exogenous SP can promote hepatocyte damage, and capsaicin depletes SP, NK1 genes in afferent neurons. Knockout or use of NK1 antagonists CP-96, 345 and L-733, 060, etc. can alleviate acute liver injury induced by various cytokines such as CD95, TNF- α [22].

In this study, animal models of hepatic ischemia-reperfusion injury were used to observe the morphology and function of hepatocytes and the expression of p-AktSer473. It was found that L-NAT can alleviate the morphological and functional destruction of hepatocytes after ischemia-reperfusion injury, increase the expression of Akt ; NK-1R receptor inhibitor L-NAT can reverse the above changes, suggesting that the hepatoprotective effect of L-NAT may be exerted by activating the PI3K-Akt signaling pathway.

In summary, this study confirmed that rat L-NAT has a protective effect on rat HIRI through a rat model of ischemia-reperfusion injury. The mechanism of action is mainly through the PI3K-Akt signaling pathway, which provides clinical application. The new basis, however, whether L-NAT has other mechanisms of action still needs further study.

5. Conclusion

L-NAT has protective effect on hepatic ischemia-reperfusion injury, moreover the protective effect of L-NAT is achieved by activating the PI3K-Akt signal transduction pathway.

Conflict of interest

No reported.

Acknowledgments

The study was supported by the the Natural Science Foundation of Shandong Province, China (ZR2018MC012; ZR2014HL020; ZR2014HL021), the project funding for the development of medical and health science and technology in Shandong Province (2014WS0464) and the neurologic disorders and regenerative repair lab “13th five-yesr plan” key lab of shandong higher education.

References

- [1] Abu-Amara M, Yang SY, Tapuria N, et al. Liver ischemia/reperfusion injury: Processes in inflammatory networks-a review[J]. *Liver Transplant*, Vol.16 (2010), 1016-1032.
- [2] Halladin NL. Oxidative and inflammatory biomarkers of ischemia and reperfusion injuries[J]. *Danish Medical Journal*, Vol. 62 (2015), B5054.
- [3] Lapi D, Colantuoni A. Remodeling of Cerebral Microcirculation after Ischemia-Reperfusion[J]. *Karger*, ,Vol. 52 (2015), 22-31.
- [4] Fernandez L, Heredia N, Grande L, et al. Preconditioning protects liver and lung damage in rat liver transplantation: role of xanthine/xanthine oxidase[J]. *Hepatology*, Vol. 36 (2002), 562-572.
- [5] Fernandez L, Heredia N, Peralta C, et al. Role of ischemicpreconditioning and the portosystemic shunt in the prevention of liver and lung damage after rat liver transplantation[J]. *Transplantation*, Vol. 76(2003), 282-289.
- [6] Luo DZ, Vermijlen D, Ahishali B, et al. On the cell biology of pit cells, the liver -specific NK cells[J]. *World J Gastroenterol*, Vol. 6 (2000), 546-552.
- [7] Ashkenazi A, Dixit VM. Death receptors: signaling and modulators[J]. *Science*, , Vol. 281 (1998), 1305-1308.
- [8] Vermijlen D, Luo DZ, Robaye B, et al. Pit cells (hepatic natural killer cells) of the rat induce apoptosis in colon carcinoma cells by perforin granzyme pathway. *Hepatology*, Vol. 29 (1999), 51-56.
- [9] Linkermann A, Green DR. Necroptosis[J]. *N Engl J Med*, Vol. 370 (2014), 455-465.

- [10] Kim JS, Ohshima S, Padiaditakis P, et al. Nitric oxide: a signaling molecule against mitochondrial permeability transition- and pH-dependent cell death after reperfusion[J]. *Free Radical Biology and Medicine*, Vol. 37 (2004), 1943-1950.
- [11] Ferraro-Peyret C, Quemeneur L, Flacher M, et al. Caspase-independent phosphatidylserine exposure during apoptosis of primary T lymphocytes[J]. *The Journal of Immunology*, Vol. 169 (2002), 4808-4810.
- [12] HLuedde T, Kapowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance[J]. *Gastroenterology*, Vol. 147 (2014), 765-783.
- [13] Liu ZX, Han D, Gunawan B, et al. Neutrophil depletion protects against murine acetaminophen hepatotoxicity[J]. *Hepatology*, Vol. 43 (2006), 1220-1230.
- [14] Liu ZX, Govindarajan S, Kaplowitz N. Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity[J]. *Gastroenterology*, Vol. 127 (2004), 1760-1774.
- [15] Reid AB, Kurten RC, Mccullough SS, et al. Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes[J]. *Journal of Pharmacology & Experimental Therapeutics*, Vol. 312 (2005), 509-516.
- [16] Mccord JM. Oxygen-derived free radicals in postischemic tissue injury[J]. *New England Journal of Medicine*, Vol. 312 (1985), 159-163.
- [17] Müller MJ, Vollmar B, Friedl HP, et al. Xanthine oxidase and superoxide radicals in portal triad crossclamping-induced microvascular reperfusion injury of the liver[J]. *Free Radical Biology and Medicine*, Vol. 21(1996), 189-197.
- [18] Hansson MJ, Månsson R, Morota S, et al. Calcium-induced generation of reactive oxygen species in brain mitochondria is mediated by permeability transition[J]. *Free Radical Biology and Medicine*, Vol. 45 (2008), 284-294.
- [19] Anderson CD, Pierce J, Nicoud I, et al. Modulation of mitochondrial calcium management attenuates hepatic warm ischemia-reperfusion injury[J]. *Liver Transplantation*, Vol. 11 (2005), 663-668.
- [20] Chen Q. Distinct stages of cytochrome c release from mitochondria: evidence for a feedback amplification loop linking caspase activation to mitochondrial dysfunction in genotoxic stress induced apoptosis[J]. *Cell Death & Differentiation*, Vol. 7 (2000), 227-233.
- [21] Teoh N, Field J, Sutton J, et al. Dual role of tumor necrosis factor-alpha in hepatic ischemia-reperfusion injury: studies in tumor necrosis factor-alpha gene knockout mice[J]. *Hepatology*, Vol. 39 (2004), 412-421.
- [22] Thapalia B A, Zhou Z, Lin X. Autophagy, a process within reperfusion injury: an update[J]. *International Journal of Clinical & Experimental Pathology*, Vol. 7 (2014), 8322-8341.