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ORIGINAL ARTICLE

# Proline protects liver from D-galactosamine hepatitis by activating the IL-6/STAT3 survival signaling pathway

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**Abstract** The oral administration of proline, one of the non-essential amino acids, has been shown to effectively protect the liver from D-galactosamine (GalN)-induced liver injury and to improve the survival rate. The aim of this study was to investigate the mechanism of this protective action of proline. We paid particular attention to the effect of proline on inflammatory activation, regenerative response, and the associated signal transduction in the liver. Male Fischer rats received intraperitoneal injections of GalN (1.4 g/kg) with or without the oral administration of proline (2 g/kg) 1 h before GalN treatment. Liver pathology, plasma indices of inflammation, and the level of proliferative marker in the liver were monitored. The hepatic activation of interleukin-6 (IL-6)/signal transducer and activator of transcription (STAT)-3 pathway, which is downstream of tumor necrosis factor (TNF)-α/nuclear factor-κB, was also studied. GalN induced massive inflammatory expansion in the liver, leading to a high death rate (60 %) more than 72 h after the treatment. Proline administration significantly suppressed inflammatory infiltration in the live after 48 h, which was accompanied by depletion of plasma TNF-α, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase. The mRNA expression of histone H3, a marker of proliferation, was significantly upregulated in the liver of proline-treated animals. Furthermore, IL-6/STAT-3 pathway, an anti-inflammatory and regenerative signaling pathway, was strongly activated prior to these observations, with the upregulated expression of downstream genes. These results suggest that the tissue-protective mechanism of proline involves the early activation of IL-6/STAT-3 pathway in the liver, with subsequent activation of the regenerative response and suppression of massive inflammatory activation.

**Keywords** Proline · D-Galactosamine · TNF-α · Liver · IL-6 · STAT3

**Abbreviations**

| | |
|---|---|
| GalN | D-Galactosamine |
| IL-6 | Interleukin-6 |
| STAT3 | Signal transducer and activator of transcription 3 |
| TNF-α | Tumor necrosis factor-α |
| NF-κB | Nuclear factor kappa B |
| GOT | Glutamic oxaloacetic transaminase |
| GPT | Glutamic pyruvic transaminase |
| LEC rat | Long Evans Cinnamon rat |
| SDS | Sodium dodecyl sulfate |
| XIAP | X-chromosome-linked inhibitor of apoptosis |
| MAPK | Mitogen-activated protein kinase kinase |
| FLIP | FLICE-inhibitory protein |
| ROS | Reactive oxygen species |
| POX | Proline oxidase |
| FAD | Flavine adenine dinucleotide |

## Introduction

The administration of a galactosamine to rodents results in extensive hepatocellular necrosis, accompanied by

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prominent infiltration of active macrophages into the hepatic stroma (MacDonald et al. 1987). The symptoms are accompanied by endotoxemia along with the release of TNF-α. Because, this liver injury histopathologically resembles human fulminant viral hepatitis, the biology of this animal model is well established. Endotoxin is known to induce the release of inflammatory mediators including TNF-α (Jirillo et al. 2002), and endotoxemia contributes significantly to the pathogenesis of galactosamine hepatitis (Grün et al. 1977). Fulminant hepatitis has similar symptoms, which is also accompanied by endotoxemia along with the release of TNF-α.

The TNF-α signaling induces either proliferation or apoptosis in a variety of cells, and NF-κB activation is the critical intracellular signal that determines the cell's response: survival or death. For cells to survive, the upregulation of NF-κB-dependent protective genes is essential. Xu et al. reported that the inhibition of NF-κB-dependent gene expression by actinomycin D sensitized hepatocytes to TNF-α cytotoxicity. When NF-κB-dependent gene expression is blocked, TNF-α treatment activates caspase-2, caspase-3 and caspase-8 (Xu et al. 1998).

It is well documented that the NF-κB/IL-6/STAT3 pathway plays a pivotal role in liver protection and regeneration in a variety of liver-injury models, including toxic damage induced with hepatotoxins (Kovalovich et al. 2000), ischemic liver injury (Matsumoto et al. 2006), and Fas-mediated injury (Kovalovich et al. 2001).

IL-6 is known as one of the most important initiators of the regenerative response (Cressman et al. 1996; Li et al. 2001). IL-6-deficient mice exhibit impaired liver regeneration characterized by liver necrosis and failure, a blunted DNA synthesis response in hepatocytes, no STAT3 activation, and reduced gene induction of the G1 phase. Treatment of IL-6-deficient mice with a single preoperative dose of IL-6 rescued STAT3 binding, gene expression, and hepatocyte proliferation to almost normal levels and prevented liver damage.

IL-6 activates a variety of pathways, including the MAPK pathway and the STAT3 pathway, which are associated with the acute response (Singh et al. 2006). Li et al. created liver-specific STAT3 deletion mice and reported that these mice exhibited reduced hepatocyte DNA synthesis at 40 h posthepatectomy, and lower expression level of G1-phase cyclins, such as cyclin D1 and E, in the liver. From these results, they conclude that STAT3 accounts for at least part of cell cycle progression and cell proliferation during liver regeneration (Li et al. 2002). In contrast, Moh et al. conditionally knocked out STAT3 in the liver (L-STAT3 (−/−)) and reported significantly higher mortality of L-STAT3 (−/−) mice within 24 h after 70 % hepatectomy (Moh et al. 2007). The level of hepatocyte DNA synthesis in the surviving L-STAT3 (−/−) mice was slightly lower than that in STAT3 (f/+) mice at 40 h posthepatectomy, whereas the liver mass completely recovered in the L-STAT3 (−/−) mice. They also showed deteriorated DNA synthesis along with increased infiltration of neutrophils and monocytes in the liver in a carbon tetrachloride model using L-STAT3 (−/−) mice. They concluded that STAT3 is required for survival in the acute stage after hepatectomy and plays an important role in the inflammatory reaction despite the limited role observed in the liver regeneration.

The importance of STAT3 in the inflammatory response is also supported by experiments in macrophage- and neutrophil-specific STAT3-deficient mice (Takeda et al. 1999). These mutant mice are highly susceptible to endotoxic shock, which involves the increased production of inflammatory cytokines, including TNF-α. It has been suggested that STAT3 plays a critical role in the IL-10-mediated anti-inflammatory response by macrophages and neutrophils.

The importance of IL-6/STAT3 pathway for the proliferative signaling was shown by Kirillova et al. (1999). They used LE6 cells, a growth-arrested rat liver epithelial cell line, to show that the NF-κB/IL-6/STAT3 pathway is essential for TNF-α-induced proliferation. They found that the TNF-α-induced upregulation of IL-6 mRNA expression and STAT3 activation was dependent on the NF-κB activation and was essential for DNA replication.

Mori et al. previously found that pre-administration of proline could counteract galactosamine hepatitis by suppressing plasma glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) elevation and significantly decreasing the mortality rate (Ajinomoto Co., Inc. 1996). They also found that proline administration delayed the onset of jaundice and subsequent death in LEC rats, which spontaneously develop lethal fulminant hepatitis due to excessive copper accumulation in the liver (Hawkins et al. 1995).

To investigate the mechanism of the protective effect of proline on GalN-induced hepatitis, we investigated the effect of proline on the activation of inflammation, the proliferative response, and the relevant survival signaling in the liver.

We found that proline administration activated the regenerative response and alleviated subsequent inflammation with the significant activation of survival signaling in the liver during the early phase of the disease.

## Materials and methods

### Animals and experimental design

Nine-week-old male Fischer 344 rats (Charles River Laboratory, Japan), weighing from 180 to 200 g, were

maintained at 23 °C, given standard laboratory chow and water ad libitum, and kept under a 12 h light (7:00–19:00)/12 h dark schedule. Inbred rat, Fischer 344, was chosen because of the large individual difference of liver damage induced by GalN in case of outbred rats. All animals received humane care in accordance with the Japanese guidelines for animal experimentation (Japanese Association for Laboratory Animal Science). All procedures used in animal experiments were approved by the Animal Ethics Committee of the Institution. Before the start of the experiment, food was withdrawn for 15 h, but the rats had free access to a 10 % glucose solution. The animals were divided to two groups, each of which consisted of five animals. Only in the case of TNF-α experiment, each group consisted of four animals. The control group received a single intraperitoneal injection of 30 % GalN saline solution between 9:00 and 10:00 am at a dose of 1.4 g/kg body weight, and the proline group received a 10 % proline solution orally at a dose of 2 g/kg body weight 1 h before GalN administration. Pre-administration of proline was determined based on the report that it is more effective than administration after GalN treatment (Ajinomoto Co., Inc. 1996).

Blood was collected from the inferior vena cava of anesthetized rats at the indicated time points after GalN treatment. After animals were killed by exsanguination at each time point, the livers were harvested and rinsed in ice-cold saline. A part of the liver was immediately frozen in liquid nitrogen for mRNA quantification and western blot analysis, and the rest of the liver was used for histological analysis.

### Survival rate

The survival rate was quantified twice per day between 8:00 and 9:00, and between 17:00 and 18:00 for up to 14 days after GalN injection.

### Measurement of GOT, GPT and TNF-α levels

The collected blood samples were centrifuged to obtain plasma. The plasma GOT and GPT levels were measured using a FUJI DRI-CHEM analyzer (Fuji Film Corporation, Japan), and the plasma TNF-α level was measured using a rat TNF-α Ultra-Sensitive Kit (BIOSOURCE International, CA, USA).

### Histology

The liver was fixed in 10 % formalin and embedded in paraffin. The blocks were sectioned at 4 μm and stained with hematoxylin–eosin according to the standard protocol. Because several animals in the control group died at 72 h after the GalN injection, the surviving animals were used at this time point.

### Isolation of total RNA and cDNA synthesis

Total RNA was isolated from liver tissue with ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Total RNA was used as the template for cDNA synthesis with Superscript II reverse transcriptase (Invitrogen, CA, USA).

### Real-Time PCR

The specificity of the PCR amplification with each primer pair was electrophoretically confirmed with a 4 % NuSieve 3:1 agarose gel (Cambrex Corporation, USA). The PCR were carried out in 20 μl reaction volumes using the SYBR Green PCR Master Mix (Applied Biosystems, USA) with 600 nM oligonucleotide primers and cDNA reverse-transcribed from 10 or 40 ng total RNA. For signal detection, the ABI Prism 7700 sequence detector (Life Technologies, CA, USA) was programmed to execute an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 thermal cycles of 15 s at 95 °C and 1 min at 60 °C. The amount of the target gene was determined using a calibration curve that was constructed using serial dilutions of the target gene. The level of mRNA was expressed as the expression level relative to the average for the control group at 0 h, which was set to 1.0.

### Western blot analysis

Liver tissue was homogenized in a lysis buffer (20 mM Tris–HCl, pH 8.0, 1 % Triton X-100, 150 mM NaCl, 10 % glycerol, 1 mM NaF, and 1 mM EDTA) containing a protease inhibitor and a phosphatase inhibitor and centrifuged at 13,000 rpm for 20 min at 4 °C. The protein concentration of the supernatant was calculated using the BCA protein assay (Pierce, IL, USA). An aliquot of protein was resolved by SDS–polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and probed with the primary antibody. Membranes were washed and probed with horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized by enhanced chemiluminescence using the ECL Plus Western Blotting Detection Reagent (GE Healthcare, CT, USA).

### Expression of data and statistical analysis

The results are expressed as the mean ± SEM. The Student's $t$ test was used for the comparison of data from the two groups. The difference between groups was considered significant when $P < 0.05$.

## Results

### The effect of proline on the inflammatory response in the liver

Hematoxylin–eosin staining of liver sections was performed to observe the infiltration of inflammatory cells and expansion of the necrotic area, and the correlation with the survival rate was determined. First, the survival rate was compared between the control and the proline groups (Fig. 1a). All of the control animals survived to 48 h after GalN administration, but the survival rate decreased to 60 % at 72 h and to 40 % at 80 h, after which point all the remaining animals survived. In contrast, no animals in the proline group died.

Representative images of the hematoxylin–eosin staining of the liver sections from the two groups are presented in Fig. 1b. In the control animals, a small number of focal necrotic areas started to appear at 6 h. The necrotic areas grew extensively between 24 and 48 h and further expanded at 72 h. The infiltration of inflammatory cells gradually became evident along with expansion of the necrotic areas. In the proline group, a few focal necrotic areas appeared as early as 3 h, at which point no sign of cell death was found in the control group. Although these early necrotic areas had grown to some extent by 24 h, they almost disappeared in association with the disappearance of infiltrated inflammatory cells by 48 h. These results suggest that proline preadministration protects the liver from GalN-induced hepatotoxicity by suppressing inflammatory activation, and as a result, significantly increased the survival rate.

### Effects of proline on the plasma GOT, GPT and TNF-α levels

To quantitatively assess the effect of proline on inflammatory activation, we examined the elevation of the plasma levels of GOT, GPT and TNF-α in GalN-treated rats.

In both control and proline groups, GalN injection caused marked elevations in the plasma GOT and GPT levels as early as 3–6 h after GalN injection. The plasma GOT and GPT levels in the proline group started to increase earlier than in the control group, peaked at 24 h, and then declined at 48 h, in contrast to the continuous elevation in the control group (Fig. 2a, b) The plasma GOT and GPT levels in the proline group at 48 h was statistically significantly lower than that of the control group ($P < 0.05$).

The plasma TNF-α levels in both groups increased markedly as early as 3 h and peaked at 6 h (Fig. 2c). Similar to the GOT and GPT profiles, elevations of TNF-α in the proline group preceded those in the control group and the level at 3 h was significantly higher ($P < 0.05$). Although differences in plasma TNF-α levels between the two groups were not statistically significant at any time

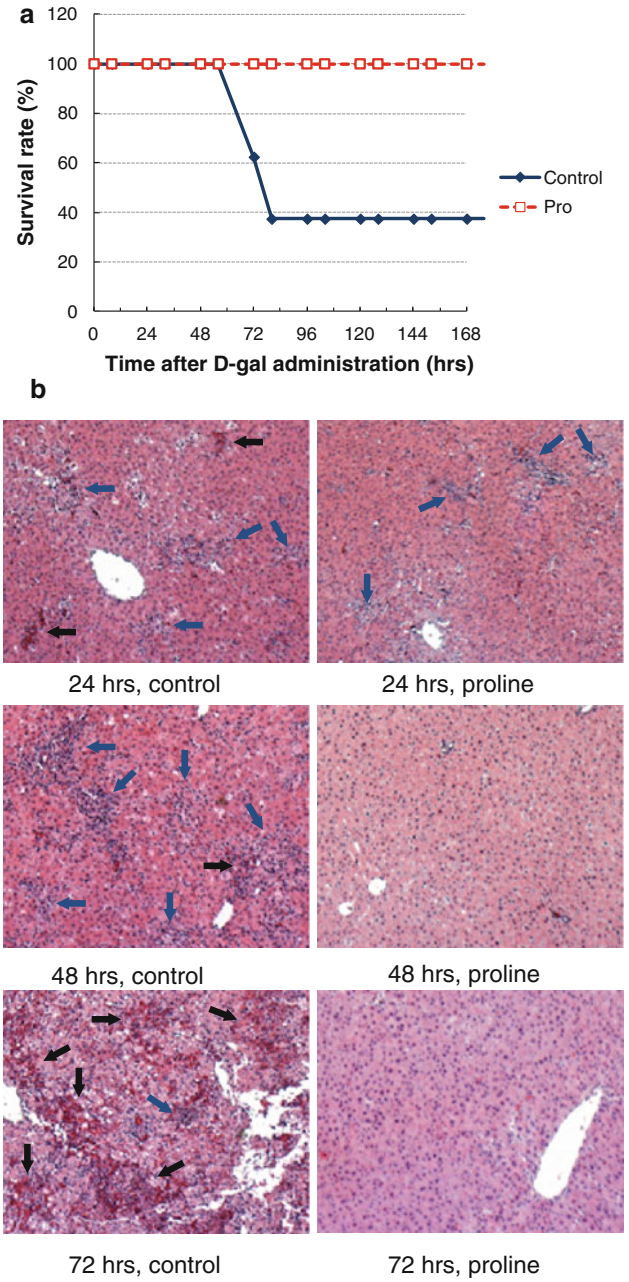


**Fig. 1** Survival rate and histology of the liver sections after GalN administration. **a** Time course of the survival rate. All animals were injected with GalN (1.4 g/kg body weight) intraperitoneally. The survival rate was quantified twice per day in the morning (8:00–9:00) and in the late afternoon (17:00–18:00). Time course of control and proline groups is denoted by *solid line* and *dotted line*, respectively. **b** The histology of the rat liver sections at 24, 48, 72 h. Hematoxylin–eosin staining was performed to observe the inflammatory infiltrates. The *blue arrows* indicate the area which is infiltrated by lot of inflammatory cells. The *black arrows* indicate necrotic area which shows the deletion of hepatocytes and the infiltration of red blood cells. (original magnification, ×100)

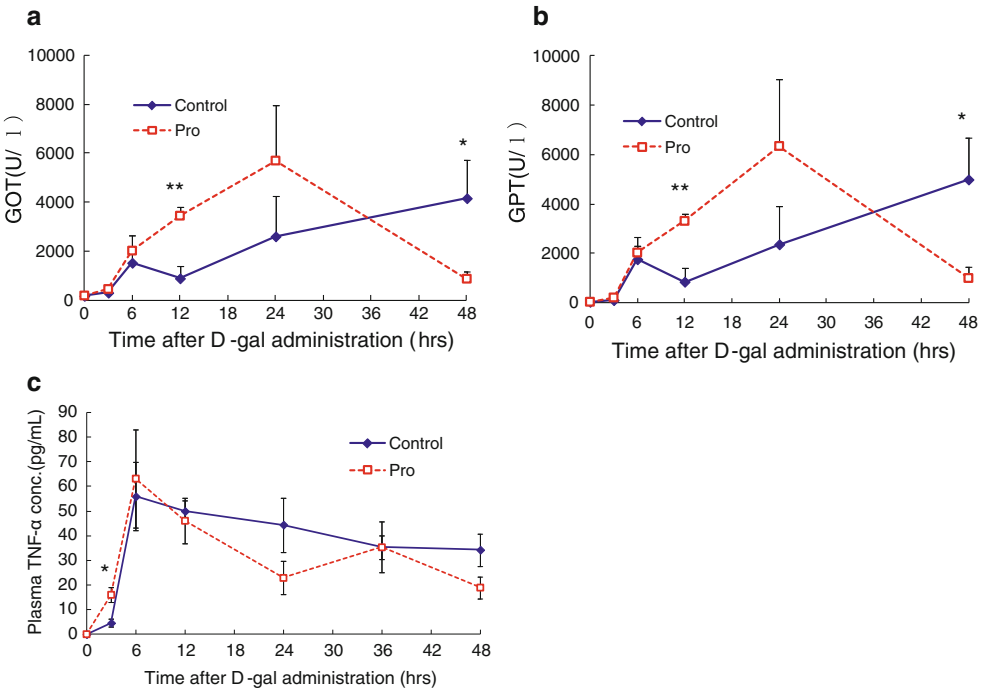


**Fig. 2** Plasma GOT, GPT and TNF-α after GalN administration. Time course of the plasma GOT (**a**), GPT (**b**) and TNF-α concentrations (**c**). Blood was collected from the inferior vena cava of the anesthetized rats at the indicated time points after GalN administration. Results are mean ± SEM. Time course of control and proline groups is denoted by *solid line* and *dotted line*, respectively. A significant difference between the two groups is denoted by *asterisk* (*$P < 0.05$, **$P < 0.01$)

point after 12 h due to the high variability among individual animals for the number of animals ($n = 4$), the mean TNF-α levels in the proline group tended to be lower at 24 and 48 h ($P = 0.10$ at 48 h).

These results suggest that proline pre-administration suppresses the predominant elevation of the plasma GOT, GPT at 48 h and TNF-α levels at 24 h and later, despite the preceding elevation seen in the earlier phase.

## Proline-enhanced proliferation in the liver

To investigate the effect of proline on the regenerative response in the liver, we examined the expression of histone H3, a marker of cell proliferation, in the liver (Vemura et al. 1992). Histone H3 mRNA is expressed during DNA replication, and this expression is tightly coupled with DNA synthesis. Histone H3 expression was significantly upregulated by approximately 8-fold at 24 h in the proline group relative to the control group [5.23 ± 2.63 vs. 41.79 ± 7.86 ($P < 0.01$), control vs. proline group] (Fig. 3). In contrast, only a marginal increase in histone H3 expression was observed in the control group prior to the 48 h time point.

## Proline-mediated activation of the IL-6/STAT3 pathway

The protective signaling of the IL-6/STAT3 pathway, downstream of TNF-α/NF-κB, has been reported to play a crucial role in the anti-inflammatory response (Moh et al. 2007; Takeda et al. 1999) and proliferation (Kirillova et al.

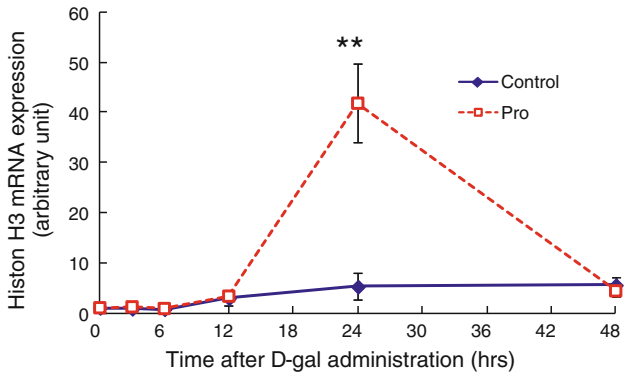


**Fig. 3** Hepatic expression of histone H3 mRNA after GalN administration. The level of mRNA is expressed as the expression level relative to the average for the control group at 0 h. Results are mean ± SEM. Time course of control and proline groups is denoted by *solid line* and *dotted line*, respectively. A significant difference between the two groups is denoted by *asterisk* (**$P < 0.01$)

1999; Cressman et al. 1996; Li et al. 2001, 2002; Xu et al. 1998; Taub 2003). Therefore, we examined the elevation of IL-6 mRNA levels and STAT3 activation in the liver.

IL-6 mRNA expression in the liver from both groups dramatically increased between 3 and 6 h after GalN treatment (Fig. 4a), and further increased in the proline group at 12 h, while that in the control group dramatically declined. The levels of IL-6 expression at 12 h in the control and the proline groups, were 208.3 ± 134.6 (an arbitrary unit) and 2,998.5 ± 1,907.5, respectively, with levels 14 times higher in the proline group ($P < 0.01$) despite substantial individual variations.

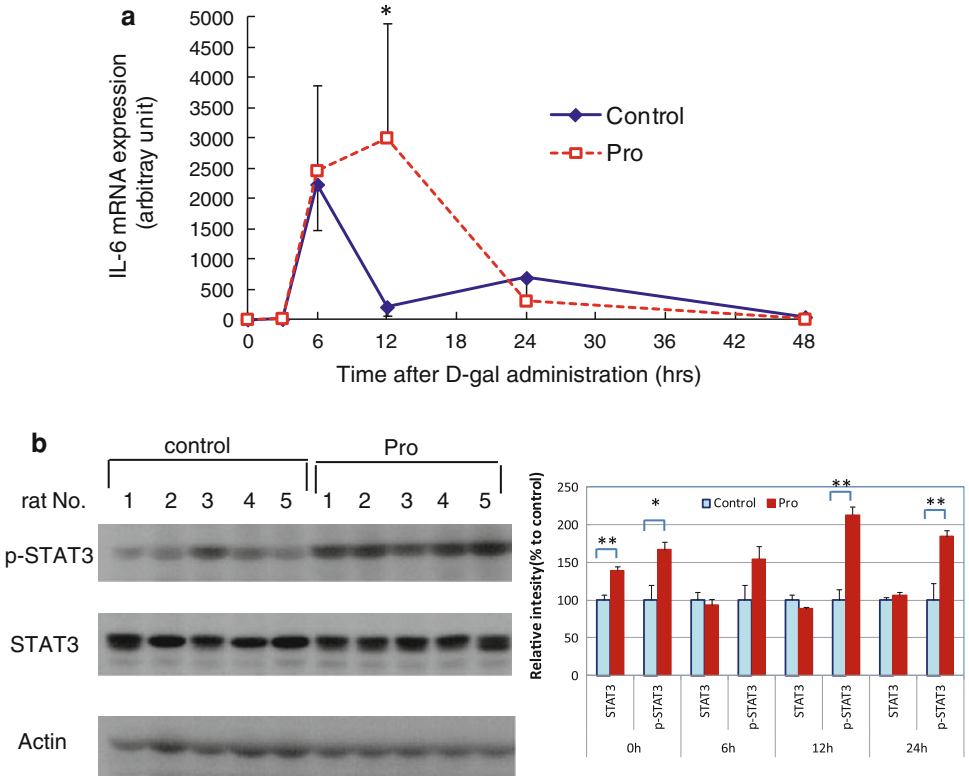


**Fig. 4** Hepatic expression of IL-6 mRNA, STAT3, and p-STAT3 proteins after GalN administration. **a** Time course of hepatic expression of IL-6 mRNA. The level of mRNA is expressed as the expression level relative to the average for the control group at 0 h. Results are mean ± SEM. Time course of control and proline groups is denoted by *solid line* and *dotted line*, respectively. A significant difference between the two groups is denoted by *asterisk* (**$P < 0.01$). **b** Western blot of STAT3, p-STAT3(Tyr705), and actin from the livers of 5 rats in each group at 12 h (*left panels*), and densitometric determination of the protein levels of STAT3 and p-STAT3 (Tyr705) (*right panel*). The protein levels of STAT3 and p-STAT3(Tyr705) was determined by western blot and expressed as the density of blot bands relative to the average of those of control rats at each time point. Results are mean ± SEM. A significant difference between the two groups is denoted by *asterisk* (*$P < 0.05$, **$P < 0.01$)

We also examined the activation of STAT3, a downstream target of IL-6, in liver tissue. The phosphorylation of Tyr705, which is essential for the activation of STAT3 (Schuringa et al. 2001), was detected and quantified by western blot at time points up to 24 h (Fig. 4b).

The intensity of each band was calculated as the intensity relative to the control group average at each time point and expressed as a percentage. At 0 h, 1 h after the proline treatment and immediately before GalN injection, the levels of both STAT3 and p-STAT3 protein were significantly higher in the proline group [STAT3 100.0 ± 7.4 % vs. 139.4 ± 5.3 % ($P < 0.01$), p-STAT3 100.0 ± 20.0 % vs. 167.1 ± 9.8 % ($P < 0.05$), control vs. proline group]. After 6 h, no difference in STAT3 levels was observed between the two groups. In contrast, p-STAT3 levels remained higher in the proline group, with the maximum difference being a two times higher at 12 h [100.0 ± 14.3 % vs. 213.3 ± 10.8 % ($P < 0.01$), control vs. proline group], and 1.8 times higher at 24 h [100.0 ± 22.3 vs. 184.5 ± 8.3 ($P < 0.01$), control vs. proline group].

In summary, these results demonstrate that proline pre-administration promotes the activation of the STAT3 transcription factor both directly and indirectly via IL-6 mRNA upregulation.

## Proline enhanced the expression of target genes of STAT3 and NF-κB transcription factors

The activation of the transcription factor STAT3, a downstream of TNF-α/NF-κB/IL-6 signaling, is involved in cell protection and proliferation, in combination with other transcription factors, such as NF-κB. In order to confirm the activation of STAT3 and NF-κB, and to investigate the hepatoprotective and regenerative effects of these transcription factors, the expression levels of genes regulated by STAT3 and/or NF-κB were assessed by real-time PCR.

The antioxidant protein Ref-1 and the antiapoptotic protein XIAP are targets of STAT3 and NF-κB, respectively, and mediate anti-apoptotic effects. The expression

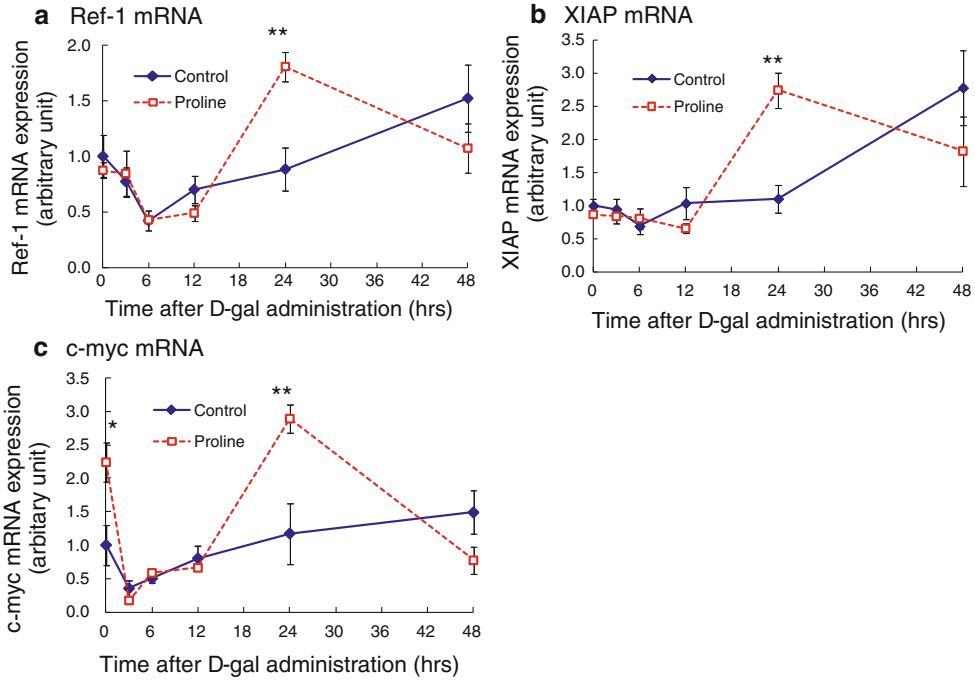


**Fig. 5** Hepatic expression of genes downstream of STAT3 and NF-κB. Hepatic expression of Ref-1 mRNA (**a**), XIAP mRNA (**b**), and c-myc mRNA (**c**). The level of mRNA is expressed as the expression level relative to the average for the control group at 0 h. Results are mean ± SEM. Time course of control and proline groups is denoted by *solid line* and *dotted line*, respectively. A significant difference between the two groups is denoted by *asterisk* (*$P < 0.05$, **$P < 0.01$)

levels of both Ref-1 and XIAP were significantly upregulated in the proline group at 24 h after GalN administration [Ref-1 $0.88 \pm 1.93$ vs. $1.81 \pm 0.13$ ($P < 0.01$), XIAP $1.10 \pm 0.21$ vs. $2.74 \pm 0.26$ ($P < 0.01$), control vs. proline group], although there was no difference between control and proline groups at time points up to 12 h (Fig. 5a, b). After the peak at 24 h, the expression levels of both molecules declined in the proline group, in contrast to the gradual increase up to 48 h observed in the control group.

The proto-oncogene c-myc is involved in cell proliferation and is regulated by various transcription factors, including STAT3 and NF-κB. Higher expression of c-myc mRNA was observed in the proline group at 0 h, 1 h after proline administration and immediately before GalN injection (Fig. 5c). Although the expression in the proline group declined and stayed at the same level as that observed in the control group between 3 and 12 h, it was significantly upregulated at 24 h [$1.17 \pm 0.45$ vs. $2.89 \pm 0.21$ ($P < 0.01$), control vs. proline group], in contrast to the marginal increase observed up to 48 h in control animals.

These results suggest that the upregulation of Ref-1 and XIAP expression correlated well with IL-6/STAT3 activation, with a significant difference in IL-6 expression level at 12 h, and in STAT3 activation at 12 and 24 h. Notably, the upregulation of c-myc mRNA correlated well with STAT3 activation, which was promoted by proline directly at 0 h and indirectly via IL-6 mRNA upregulation between 6 and 12 h.

## Discussion

### Proline suppressed inflammatory activation and activated the regenerative response probably via IL-6/STAT3 pathway

Mori et al. reported that the oral administration of proline improved the mortality rate in a GalN-induced model of liver failure by suppressing the plasma GOT and GPT elevation (Ajinomoto Co., Inc. 1996). To investigate the mechanism of the protective effect of proline on this model, we examined the inflammatory response and regenerative activation, which are crucial to hepatic survival. We found that proline administration induced early hepatic upregulation of the histone H3 mRNA (a proliferative marker) at 24 h and decreased the inflammatory infiltration in the liver after 48 h in association with the fewer necrotic area, the significant decrease of plasma GOT, GPT, and the lower trend of plasma TNF-α. These results suggest that the early regenerative activation and the suppression of fulminant inflammation play important roles in the hepatoprotective effect of proline. We then hypothesized that survival signaling downstream of TNF-α might be upregulated by proline.

It is well known that TNF-α is a mediator of the hepatic acute-phase response to inflammation and induces both cell death and proliferation. D-Galactosamine injection induces serum TNF-α elevation, which triggers hepatocyte apoptosis (Itokazu et al. 1999). The activation of NF-κB triggered by TNF-α is essential for cell survival in the acute

phase of injury mediated by hepatic toxins, ischemia–reperfusion and partial hepatectomy. Furthermore, the IL-6/STAT3 pathway, downstream of TNF-α/NF-κB, plays an important role in this protective mechanism.

In this study, we found that proline administration activated the IL-6/STAT3 pathway within 24 h after GalN treatment. IL-6 mRNA upregulation was sustained for a longer time in the liver of proline-treated rats, which exhibited 14-fold higher expression at 12 h, compared with that of the control rats. The level of p-STAT3 (Tyr705) was two times higher at 12 h (maximum difference) and 1.8 times higher at 24 h.

We observed that the inflammatory infiltration was profoundly suppressed in the liver of proline-treated animals at 48 h in contrast to that of control animals, which showed predominant inflammatory infiltration at the same time point with further increase of infiltration up to 72 h. This result is supported by the early decrease of plasma GOT, GPT, and TNF-α in proline group at 48 h, despite the continuous increase of plasma GOT and GPT in control group. These results suggest the importance of STAT3 activation in the suppression of inflammatory activation.

In addition to the early suppression of inflammatory activation, we also found that proline administration markedly induced the mRNA expression of a proliferation marker, histone H3, in the liver at 24 h after GalN treatment. The downstream targets of IL-6, MAPK and STAT3 work collaboratively to enhance cell proliferation. Recently, it is also reported that the IL-6/STAT3 pathway regulates hepatocyte proliferation via cyclin D1/p21 (Fujiyoshi and Ozaki 2011). Therefore, the longer period of upregulation of IL-6 mRNA induced by proline from 6 to 12 h should contribute to the regenerative activation.

In addition to proliferative and anti-inflammatory effects, STAT3 plays an important role in preventing apoptosis through the induction of antioxidant proteins, such as Ref-1, and antiapoptotic proteins which block caspase activation (Haga et al. 2003). Recently, it has also been reported that STAT3 interacts with NF-κB and enhances NF-κB-dependent gene induction (Lee et al. 2009). Notably, STAT3 prolongs NF-κB nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF-κB nuclear export. Reversible acetylation of RelA regulates the duration of nuclear NF-κB activity (Chen et al. 2001). We observed that the antioxidant protein Ref-1 (a known target of STAT3), the antiapoptotic protein XIAP (a known target of NF-κB), and the proto-oncogene c-myc (a known target of both STAT3 and NF-κB) are all significantly increased in the liver of proline-treated animals at 24 h after GalN injection. This timing of mRNA upregulation correlated well with that of IL-6 between 6 and 12 h and p-STAT3 between 12 and 24 h. These results confirm the upregulation of the IL-6/STAT3 pathway in the proline group, and the induction of expression of these genes may also contribute to the hepatic antiapoptotic response and cell proliferation.

Proline may protect the liver in another way. As shown in Fig. 4b, proline administration itself, even without GalN injection, directly upregulated the levels of STAT3 and p-STAT3 proteins in the liver. The amount of both proteins was significantly higher at 0 h, that is, 1 h after proline administration and immediately before GalN injection. Based on the report that the administration of proline prior to GalN treatment is more effective than the administration after GalN treatment (Ajinomoto o., Inc. 1996), this result suggests that the direct activation of STAT3 without the upregulation of IL-6 mRNA should also contribute to the tissue-protective effect mediated by proline.

In summary, we found that proline administration enhanced the hepatic regenerative response and suppressed inflammatory activation. These effects are thought to be triggered by prior activation of IL-6/STAT3 pathway, and play a crucial role in the hepatoprotective effect of proline.

## Why is the IL-6/STAT3 pathway specifically upregulated by proline administration?

We found that IL-6 mRNA expression downstream of TNF-α signaling was dramatically increased in the liver by proline administration. Although the source of IL-6 within the liver has not been unequivocally established, studies with bone marrow transplantation provide evidence that hepatic Kupffer cells (liver macrophages) are responsible for the production of IL-6 in response to lipopolysaccharide or TNF-α (Aldeguer et al. 2002). The transcription factor NF-κB is critical for the induction of IL-6 mRNA expression, and ROS is reported to activate NF-κB in response to TNF-α in immune cells (Gloire et al. 2006). Furthermore, TNF-α itself is also regulated by NF-κB in immune cells during the inflammatory response.

We found that the elevation of serum TNF-α preceded in the proline group, with the significant difference at 3 h with that of control group. We also found that catalase activity was significantly enhanced in the proline group already at 0 h, which was just before GalN injection (data not shown). Based on this finding, we hypothesized that proline administration increases the production of ROS in the mitochondria before the GalN injection, and that increased ROS worked as a preconditioning before severe inflammation caused by GalN injection.

The first enzyme involved in proline metabolism is proline oxidase (POX), which is tightly bound to the mitochondrial inner membrane and a rate-limiting enzyme in proline degradation. Proline oxidase converts proline to Δ1-pyrroline-5-carboxylate, which serves as an obligate carbon bridge between the two major metabolic cycles, the

tricarboxylic acid cycle and the urea cycle (Phang 1985; Wu et al. 2011). The catalytic mechanism involves the transfer of electrons from substrate proline to FAD with cytochrome *c* as the subsequent carrier into the electron transport chain. Thus, proline is a direct substrate for the generation of ATP (Hagedorn and Phang 1983; Adams and Frank 1980, 1983). Recently, it was shown that POX can reduce oxygen and generate superoxide (Donald et al. 2001). The FAD of POX has direct access to solvent oxygen, and proline-derived electrons can directly reduce oxygen to produce superoxide autogenously.

Regarding ischemia/reperfusion (I/R) injury, it is well known that ROS production and the subsequent secretion of TNF-α significantly contributes to the injury, and short-term ischemia increases resistance to subsequent lethal ischemia/reperfusion with the suppression of massive inflammatory activation. Teoh et al. (2003) reported that following the ischemic preconditioning stimulus, there was an early small rise in hepatic and serum TNF-α levels, but during a next prolonged ischemia when TNF-α was secreted more than that during preconditioning, TNF-α release was lower and declined to negligible level earlier compared with naïve mice (Teoh et al. 2003). This protective effect of preconditioning is mimicked by the pre-administration of single low dose of TNF-α. These results suggest that the small rise of plasma TNF-α triggered by short ischemia plays an essential role in the protective effect of preconditioning. It is also reported that both preconditioning and pre-administration of TNF-α induced early activation of NF-κB and STAT3 transcription factors and subsequent increase of hepatic cyclin D1 protein expression and PCNA positive nuclei. Although the exact mechanism of preconditioning has not yet been elucidated, the protective effect of proline is very similar to that observed in preconditioning model of I/R injury, such as small rise of ROS and TNF-α before lethal rise of plasma TNF-α.

Our hypothesis is that the ROS produced by proline administration may accelerate the activation of the NF-κB at very early phase, and subsequent small rise of plasma TNF-α at 3 h after GalN injection may also make a positive effect on the extended activation of NF-κB. Enhanced activation of NF-κB, especially in the immune cells including Kupffer cells, should have contributed to the higher and prolonged activation of IL-6/STAT3 pathway and early regenerative response in the proline group. Although the prior increase of plasma TNF-α enhanced the activities of plasma GOT and GPT at 24 h compared with control animals, the activation of regenerative response at the same time point should counteract the damage in the liver.

In conclusion, this study has demonstrated that the protective mechanism mediated by proline in GalN-induced hepatitis is attributable to the early regenerative response and early reduction of inflammation, which is thought to be triggered by prior activation of the IL-6/STAT3 pathway, downstream of TNF-α/NF-κB signaling in the liver. The generation of ROS through proline metabolism by POX in mitochondria and early small rise of plasma TNF-α may underlie the enhanced activation of the NF-κB transcription factor. These results further emphasize the potential utilization of proline in protecting the liver against drug-induced injury, endotoxin shock, and ischemia/reperfusion injury including liver transplantation, in which TNF-α triggers liver failure.

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**Conflict of interest** This study was supported by a private fund from Ajinomoto Co., Inc. All authors are employee of Ajinomoto Co., Inc., which has published a large number of papers about the function of amino acids, strenuously doing basic research. No patent has been applied based on the research of this manuscript.

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