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• BASIC RESEARCH •

The role of endotoxin, TNF-α, and IL-6 in inducing the state of growth hormone insensitivity

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Abstract

AIM: **Critical illnesses such as sepsis, trauma, and burns cause a growth hormone insensitivity, which leads to an increased negative nitrogen balance. Endotoxin is generously released into blood under these conditions and stimulates the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1, which may play a very important role in inducing the growth hormone insensitivity. The objective of this current study was to investigate the role of endotoxin, TNF-α and IL-6 in inducing the growth hormone insensitivity at the receptor and post-receptor levels.**

METHODS: **Spague-Dawley rats were injected with endotoxin, TNF-α, and IL-6, respectively and part of rats injected with endotoxin was treated with exogenous somatotropin simultaneously. All rats were killed at different time points. The expression of IGF-I, GHR, SOCS-3 and β-actin mRNA in the liver was detected by RT-PCR and the GH levels were measured by radioimmunoassay, the levels of TNF-α and IL-6 were detected by ELISA.**

RESULTS: **There was no significant difference in serous GH levels between experimental group and control rats after endotoxin injection, however, liver IGF-I mRNA expression had been obviously down-regulated in endotoxemic rats. Liver GHR mRNA expression also had a predominant down-regulation after endotoxin injection. The lowest regulation of liver IGF-I mRNA expression occurred at 12h after LPS injection, being decreased by 53% compared with control rats. For GHR mRNA expression, the lowest expression occurred at 8h and had a 81% decrease. Although SOCS-3 mRNA was weakly expressed in control rats, it was strongly up-regulated after LPS injection and had a 7.84 times increase compared with control rats. Exogenous GH could enhance IGF-I mRNA expression in control rats, but it did fail to prevent the decline in IGF-I mRNA expression in endotoxemic rats. Endotoxin stimulated the production of TNF-α and IL-6, and the elevated IL-6 levels was shown a positive correlation with increased SOCS-3 mRNA expression. The liver GHR mRNA expression was obviously down-regulated after TNF-α iv injection and had a 40% decrease at 8h, but the liver SOCS-3 mRNA expression was the 4.94 times up-regulation occurred at 40min after IL-6 injection.**

CONCLUSION: **The growth hormone insensitivity could be induced by LPS injection, which was associated with down-regulated GHR mRNA expression at receptor level and with up-regulated SOCS-3 mRNA expression at post-receptor level. The in vivo biological activities of LPS were mediated by TNF-α and IL-6 indirectly, and TNF-α and IL-6 may exert their effects on the receptor and post-receptor levels respectively.**

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INTRODUCTION

Infection especially severe intra-abdominal infection is characterized by catabolic status associated with severe protein loss and negative nitrogen balance[1-7]. Meantime, the levels of many important hormones such as glucocorticoid, insulin and growth hormone (GH) do not decline, but their biological activities have reduced obviously. Critical illnesses such as sepsis, trauma and burns can usually cause a elevated level of growth hormone at early stage, however the insulin-like growth factor I (IGF-I), which is a growth hormone-dependent growth factor that inhibits protein breakdown, has been showing decreased predominantly, this phenomenon indicating a status of growth hormone insensitivity[8-12]. In this condition, the administration of high doses of recombinant human growth hormone could not improve negative nitrogen balance, in contrary, it may lead to other metabolic disorders and result in increased morbidity and mortality[13].

Endotoxin is generously released into blood under the infected condition and stimulates the production of proinflammatory cyotkines such as TNF-α, IL-6, and IL-1[14-18], which play very important roles in inducing the GH insensitivity. The GH insensitivity can occur at receptor and post-receptor levels, the receptor level associates with down-regulated GHR mRNA expression[19, 20]; the post-receptor insensitivity mainly occurs on the intracellular signal transduction pathway of growth hormone. Recent studies have suggested that the SOCS protein family, especially SOCS-3 play a very important role on this level[21,22]. In this study, we investigated whether the GH insensitivity could be induced by LPS, TNF-α and IL-6 iv injection, and what kind of roles they played.

MATERIALS AND METHODS

Animals

All experimental procedures were carried out in compliance with the appropriate institutional and national ethical guidelines for work with laboratory animals. 156 adolescent male Spague-Dawley rats (240-260g) were obtained from animal center of Jinling Hospital (Nanjing, China). They were given free access to food and water for three days before experiments.

Endotoxin and cytokines preparation

Escherichia coli lipopolysaccharide (LPS; serotype O111:B4 phenol extract), obtained from Sigma Chemical (St. Louis, MO), was resuspended in sterile endotoxin-free saline to obtain 4mg/ml

solutions. The recombinant rat TNF-α and IL-6 provided by Pepro Tech EC Ltd (London, England), were resuspended in sterile endotoxin-free saline to obtain 100000U/ml solutions. Human growth hormone, kindly provided by Serono, was resuspended in sterile endotoxin-free saline to obtain a 1mg/ml solution.

Experimental protocols

Male Spague-Dawley rats (provided by Animal Center of Jingling Hospital), weighing 250±10g, were given free access to food and water for three days before experiments. Rats were anesthetized with ether and received LPS, GH, TNF-α, IL-6, and saline injection, LPS, TNF-α, and IL-6 were administered through superficial dorsal veins of penis and GH was injected subcutaneously. All rats were killed at different time points; blood of rats with LPS injected was collected and centrifuged at 500g for 10min at 4℃ to collect serum. Livers were removed, flash-frozen in liquid nitrogen, and stored at -80℃ until homogenate preparation and RNA extraction.

Effect of endotoxin on liver expression of IGF-I, GHR, and SOCS-3 mRNA

After the 3-day adaptation period, 42 rats were randomly divided into laboratory group (n=36) and control group (n=6), LPS (7.5mg.kg^{-1} iv) was administered to the laboratory group, every six rats were killed at 1h, 2h, 4h, 8h, 12h, and 24h after injection. The control rats were given intravenous saline.

Effect of GH on liver expression of IGF-I, GHR, and SOCS-3 mRNA along with endotoxin injection

After 3-day adaptation period, 24 rats were divided into 4 groups (6 rats/group). The first group received one injection of LPS (7.5mg.kg^{-1} iv) and one injection of saline (sc), the second group received one injection of GH (1.5mg.kg^{-1} sc) and one injection of saline (iv), the third group received one injection of LPS (7.5mg.kg^{-1} iv) and one injection of GH (1.5mg.kg^{-1} sc), the fourth group received two injections of saline. All rats were killed at 10h after injection.

Effects of TNF-α and IL-6 on liver expression of GHR and SOCS-3 mRNA

After the 3-day adaptation period, 102 rats were randomly divided into laboratory group (n=96) and control group (n=6). Rat recombinant TNF-α and IL-6 (100000U/kg.wt) were injected through the same pathway and every six rats were killed after 20min, 40min, 1h, 2h, 4h, 8h, 12h and 24h. The control rats were given intravenous saline.

Analysis of mRNA by RT-PCR

Freshed-frozen liver samples were homogenized and total RNA was performed using TRIZOL Reagent (Biobasic Inc, Scarborough, Ontario, Canada). With Access RT-PCR system kit (Promega Coporation, Madison, WI), the cDNA synthesis and amplification was done in one tube following the manufacture's instructions. In brief, 1μg RNA, 1μM primers for SOCS-3, GHR, IGF-I and β-actin were added to each reaction mixture respectively, which included 0.2mM dNTP, 1mM MgSO4, AMV reverse transcriptase 5U, Tfl DNA polymerase 5U, and AMV/Tfl 5×buffer 10μL. The reaction final volume was 50μL and was covered with 30μL mineral oil. RT-PCR reaction was run in the following procedures: (1)Reverse transcription: 48℃ for 45min, 1 cycle. (2)AMV RT inactivation and RNA/cDNA/primers denaturation: 94℃ for 2min, 1 cycle. (3) Second strand cDNA synthesis and PCR amplification: denaturation 94℃ for 30s, annealing 60℃ for 1min, extension 68℃ for 2min, 28 cycles for SOCS-3 and 21 cycles for GHR and IGF-I, β-actin as intra-control to be amplified along with SOCS-3, GHR and IGF-I. (4) Final extension: 68℃ for 7min, 1 cycle. 5μL each RT-PCR reaction was electrophoresed in a 1.7% Metaphor agarose (FMC Bioproducts, Rockland, ME) gel and stained with ethidium bromide. Products of RT-PCR reactions were photographed and analyzed by densitometry. The expression of IGF-I, GHR, and SOCS-3 mRNA in laboratory group is represented as a percentage or times compared with their expression in control group.

Polymerase chain reaction primers were as follows: IGF-I sense, (5') CAC ATC TCT TCT ACC TGG CAC TC (3'); IGF-I antisense, (5') GGA TGG AAC GAG CTG ACT TTG TA (3'), to give a 270 base pair product; GHR sense, (5') CTG GGT TGA GTT CAT TGA GCT GGA T (3'); GHR antisense, (5') TGT AGA GGG GAG TTG GTG GGT TGA C (3'), to give a 394 base pair product; SOCS-3 sense, (5') ACC AGC GCC ACT TCT TCA CG (3'); and SOCS-3 antisense, (5') GTG GAG CAT CAT ACT GAT CC (3'), to give a 450 base pair product; β-actin sense, (5') CAT TTC CGG TGC ACG ATG GAG (3'); β-actin antisense, (5') GCC ATC CTG CGT CTG GAC CTG (3'), to give a 599 base pair production. All primers spanned at least one intron of genomic DNA.

Serum levels of GH, TNF-α and IL-6

Blood was obtained from the inferior vena cava at the time of sacrifice. Serum growth hormone levels was measured by radioimmunoassay according to manufacture's instructions (Northern Isotope Co, Beijing, China). Serum samples were analyzed for TNF-α and IL-6 content by enzyme-linked immunosorbent assay according to manufacture's instructions (BioSource International, Camarillo, CA).

Statistics ananlysis

All data are expressed as means ± SEM. Correlation between data was analyzed with linear regression. Comparisons between two groups were performed using an unpaired Student's t test. Differences were considered statistically significant when P<0.05.

RESULTS

Levels of serum growth hormone after endotoxin injection

The levels of serum growth hormome at each time points after LPS injection had no significant difference compared with control rats, it maintained a relatively stable status (Table1).

Table 1 Serum GH levels in LPS injected rats at different time points and control rats

	n	GH levels(ng/ml)
Control	6	2.19±0.48
1h	6	1.85±0.37[a]
2h	6	1.95±0.45[a]
4h	6	1.76±0.27[a]
8h	6	1.79±0.27[a]
12h	6	1.77±0.20[a]
24h	6	1.79±0.55[a]

[a]Statistically no difference compared with control rats.

Liver IGF-I mRNA expression

Liver IGF-I mRNA expression had already declined by 25% *vs*. control rats at 8 hours. On the time of 12 hours, we observed the lowest level of expression, which was a 53% decrease compared with control rats. It did not recover to the normal level and had a 15% reduction at 24 hours (Figure 1A, 1B). Although exogenous GH administration in control rats significantly enhanced the liver IGF-I mRNA expression, it did fail to prevent its decline in endotoxemic rats (Figure 1C).

Liver GHR mRNA expression

Liver GHR mRNA expression had already down-regulated by 45% at 2 hours after LPS injection, the lowest regulation occurred at 8 hours, which was a 89% decrease compared with control rats. After 24 hours, it did not recover to the normal level and had a 44% decrease (Figure 2A, 2B). the exogenous GH administration had no effect on the liver GHR mRNA expression in control and endotoxemic rats (Figure 2C).

Liver SOCS-3 mRNA expression

The liver SOCS-3 mRNA was weakly expressed in control rats, however, it was strongly up-regulated by 7.84 times *vs*. control rats at 1 hour after LPS injection. This level was maintained at 2 hours and it still had a 1.8 times increase at 24 hours (Figure 3A, 3B). the exogenous GH infusion had no effect on the liver SOCS-3 mRNA expression in control and endotoxemic rats (Figure 3C).

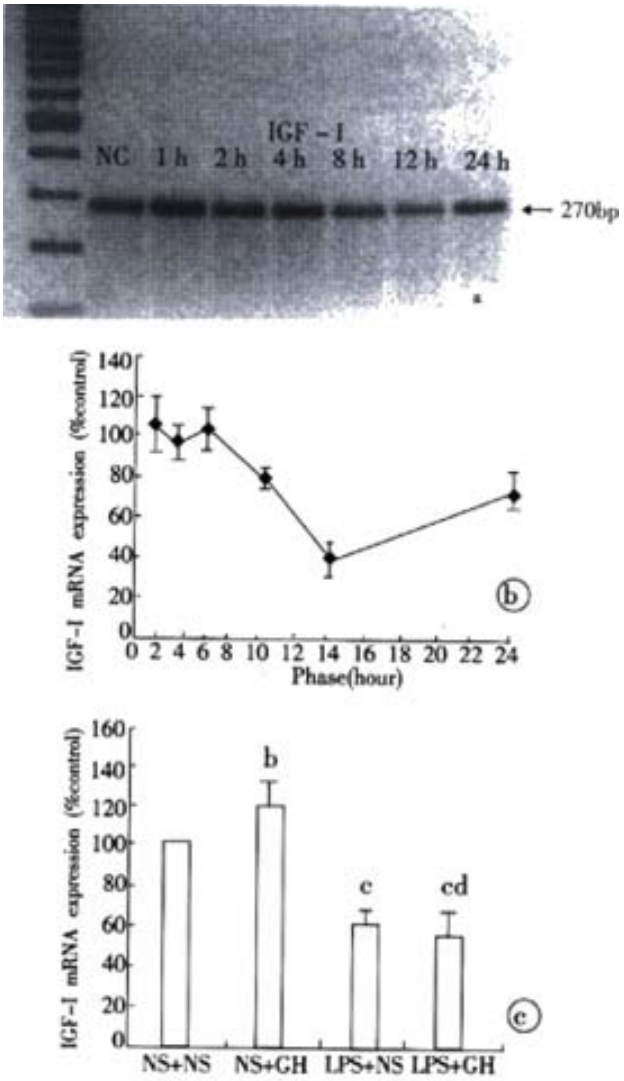

Figure 1 (A, B) Liver IGF-I mRNA expression response to endotoxin injection at different time points. (C) Liver IGF-I mRNA expression after single GH injection and endotoxin injection along with or without GH injection. [b]*P*<0.05 compared with NS+NS group, [c]*P*<0.01 *vs* NS+NS group, [d]*P*>0.05 compared with LPS+NS group. NS as saline injection.

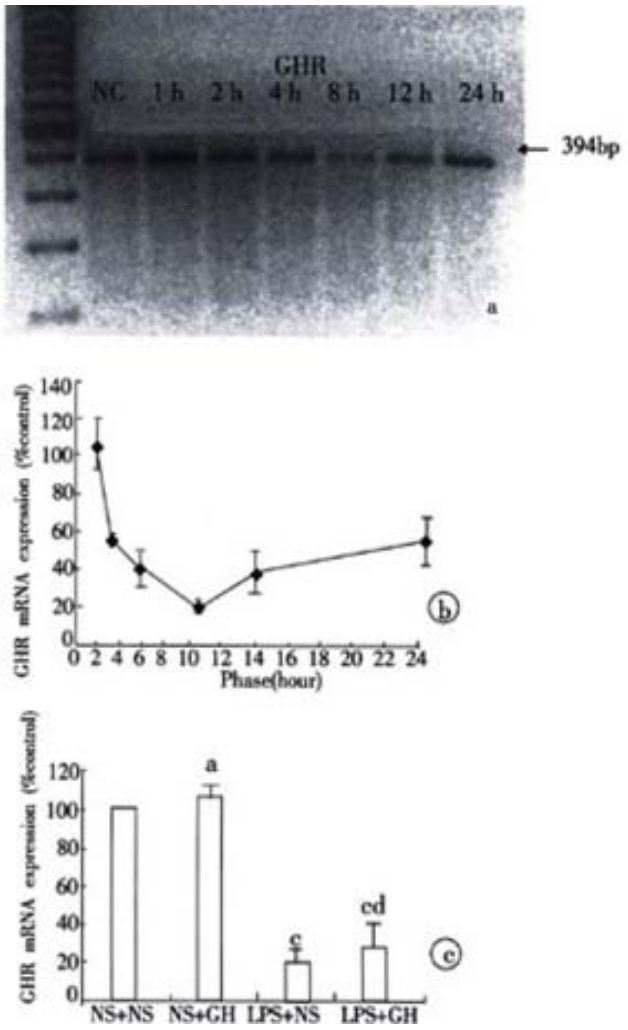

Figure 2 (A, B) Liver GHR mRNA expression responsed to endotoxin injection at different time points. (C) Liver GHR mRNA expression after single GH injection and endotoxin injection along with or without GH injection. [a]*P*>0.05 compared with NS+NS group, [c]*P*<0.01 *vs* NS+NS group, [d]*P*>0.05 compared with LPS+NS group. NS as saline injection.

Levels of serum TNF-α and IL-6 after LPS injection and the correlation between liver SOCS-3 mRMA expression and IL-6 concentration

The TNF-α level was increased rapidly after LPS injection, but it decreased obviously from the second hour and returned to the normal level at 4h. The IL-6 level was also elevated rapidly after LPS injection; it got to the highest level at 2h and then decreased gradually (Table 2). Linear regression analysis was shown a positive correlation of IL-6 with liver SOCS-3 mRNA expression (r=0.935, P<0.01).

Table 2 Serum TNF-α and IL-6 levels in LPS injected rats at different time points and control rats

	n	TNF-α levels(pg/ml)	IL-6 levels(pg/ml)
Control	6	<20	<8
1h	6	342.80±50.01	1438.74±323.07
2h	6	75.81±11.50	1678.03±126.57
4h	6	<20	1332.67±120.95
8h	6	<20	142.59±48.07
12h	6	<20	48.75±10.57
24h	6	<20	46.82±11.64

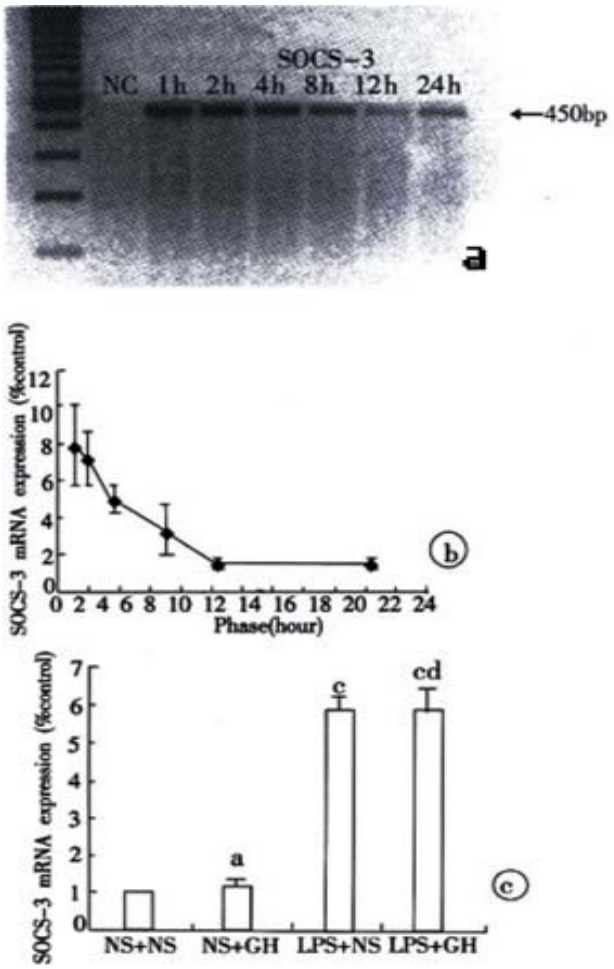

Figure 3 (A, B) Liver SOCS-3 mRNA expression response to endotoxin injection at different time points. (C) Liver SOCS-3 mRNA expression after endotoxin injection along with or without GH injection. [a]*P*>0.05 compared with NS+NS group, [c]*P*<0.01 *vs* NS+NS group, [d]*P*>0.05 compared with LPS+NS(7.5) group. NS as saline injection.

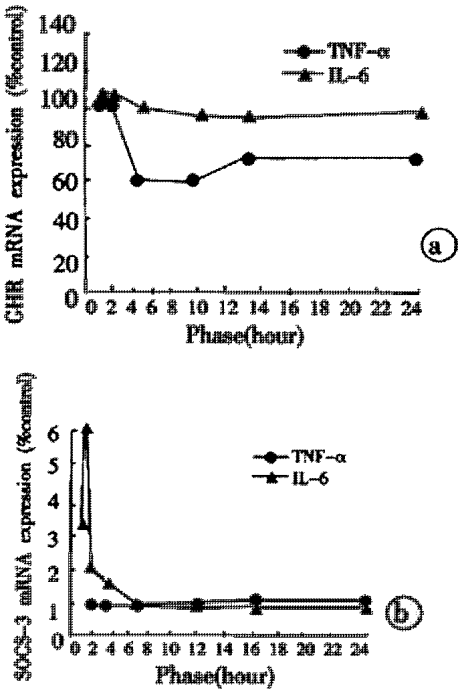

Figure 4 LiverGHR(A) and SOCS-3(B) mRNA expression response to TNF-α and IL-6 iv injection at different time points.

Effects of TNF-α and IL-6 on liver expression of GHR and SOCS-3 mRNA

The liver GHR mRNA expression after TNF-α injection had already down-regulated at 4 hours and it reached the lowest level at 8 hours, which was a 40% decrease compared with control rats. At 24 hours, a 27% reduction still existed. The IL-6 injection had no effect on the liver GHR mRNA expression at different time points (Figure 4A). The liver SOCS-3 mRNA had weak expressions at all time points after TNF-α injection, no difference could be found compared with control rats. The IL-6 injection was able to up-ragulate rapidly the liver SOCS-3 mRNA expression, the latter showing a 2.73 times increase at 20 minutes and the highest level occurred at 40 minutes with a 4.94 times increase compared with control rats (Figure 4B).

DISCUSSION

In this report, using an experimental method of *E. coli* endotoxin infusion in laboratory rats, we have found endotoxin-induced growth hormone insensitivity. At 12 hours after LPS injection, there was no difference in serous growth hormone concentration between the experimental and control rats, however, the liver IGF-I mRNA expression had already declined obviously. In control rats, the liver IGF-I mRNA expression was up-regulated by 25% after exegeous GH administration, but in endotoxemic rats, GH did fail to prevent the decline in liver IGF-I mRNA expression. Several groups have observed that decreased IGF-I may result from a state of GH insensitivity. Ross *et al*[8]. reported low circulating IGF-I levels in critically ill patients despite elevated GH secretion. More recently, the sdudy[19] showed that after a single injection of LPS in rats, plasma IGF-I level remained low despite the fact that GH level had returned to normal value. In agreement with these authors, our study support the possibility that the GH insensitivity maybe one of the important factors for the reduced liver IGF-I mRNA expression after LPS injection.

Growth hormone insensitivity can occur at receptor and post receptor levels, on the receptor level GH insensitivity is associated with the reduced GHR numbers on target cell sueface[19,20]. Because of the shorter half-life of liver GHR (30-40min)[23] and the decreased liver GHR mRNA expression by endotoxin, these led to the reduced GHR synthesis. Our results shown that liver GHR mRNA expression was obviously down-regulated after LPS injection, manifested that LPS had effect on the receptor level GH insensitivity indeed.

The factor of post-receptor level GH insensitivity has caused more and more attention recently, and it is associated with a novel family of suppressor of cytokine signalling (SOCS) which includes eight members (SOCS-1 to SOCS-7 and CIS) that act in a classical negative feedback loop to regulate cytokine signal transduction[24-29]. SOCS-3 is a strong inhibitor on growth hormone intracellular signal transduction[30-32].

Once growth hormone binds to its receptor, the intracellular signal transduction is activated through JAK-STAT pathway[33, 34]. The first activated tyrosine kinase is JAK2, which promotes the tyrosyl phosphorylation of both JAK2 itself and signal transducer and activator of transcription 5b (STAT 5b). Phosphorylated STAT 5b causes its dimerization and then the dimerized STAT 5b translocates into the nucleus, where it binds with high affinity to the promoters of various target genes and then activates the gene transcription such as IGF-I. SOCS-3 can block the GH intracellular JAK/STAT-dependent signaling pathway at different levels[35-44], including Competitively inhibits the phosphorylation of STAT 5b. Binds to GHR and leads to the degration of GHR-JAK2 compound directly or indirectly through Elongin B and Elongin C, in the end the JAK2 kinase loses its activity. Through binding to GHR, SOCS-3 can inhibit the JAK2 kinase activity directly. Our experiment observed that after LPS injection, liver SOCS-3 mRNA expression was rapidly up-regulated with a 7.84 times increase at 1 hour compared with the weak expression in control rats, this

indicating that LPS induced the production of post-receptor GH insensitivity.

The *in vivo* biological activities of LPS are largely mediated by the production of proinflammatory cytokines such as tumor necrosis factor-α(TNF-α), interleukin-1β(IL-1β), and IL-6[45-50], which is illustrated in our experiment by the marked stimulation of the secretion of TNF-α and IL-6 in blood. TNF-α and IL-6 had different roles in inducing the GH insensitivity, TNF-α injection leading to a reduced expression of liver GHR mRNA, and IL-6 being associated with the up-regulated SOCS-3 mRNA expression after injection. The elevated IL-6 levels stimulated by LPS had significant positive correlation with the increased liver SOCS-3 mRNA expression induced by LPS.

The mechanisms of TNF-α-induced GHR mRNA suppression was mostly mediated by inhibition of Sp transactivator binding to the L2 promoter of GHR gene[51], the other way might be associated with some other cytokines stimulated by TNF-α, such as IL-1[52].Both IL-6 and GH, belonging to the cytokine receptor superfamily, can transduce their signal from cell surface to nucleus through the same JAK-STAT pathway[53-55]. Hence, the elevated IL-6 levels stimulated by LPS promoted the increased expression of SOCS-3 mRNA, which not only had a negative feedback to IL-6 biological activities, but also inhibited the GH intracellular signal transduction[56-60].

In summary, our study observed that the growth hormone insensitivity could be induced by endotoxin, which suggested that the endotoxin played a very important role in inducing the GH insensitivity. The endotoxin not only had predominant effect on the GHR gene expression, but also induced the phenomenon of negative feedback loop at post-receptor level. The toxic effect of endotoxin was mostly mediated by TNF-α and IL-6 indirectly, and TNF-α maily had effect on the receptor gene expression, but for IL-6, it mainly caused the negative feedback loop at post-receptor level.

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