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ORIGINAL ARTICLE

The effects of subclinical hypothyroidism on serum lipid level and TLR4 expression of monocyte in peripheral blood of rats

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Abstract

OBJECTIVE: To observe effect of subclinical hypothyroidism (SCH) on serum lipid level and expression of toll-like receptor 4 (TLR4) in rats' peripheral blood monouclar cells (PBMC).

METHODS: Fifty Wistar female rats were divided into three groups: normal control (NC group; n=10), sham group (n=10), and L-T-4 (L-thyroxine) group (n=30, with thyroidectomy, fed with rich-calcium water after operation. 5 weeks later, abdominal subcutaneous injection of L-T-4: 0.95 μg/100g/d). 8 weeks later, the rats were killed then the peripheral blood was collected to determine the levels of serum thyroid-stimulating hormone (TSH), total thyroid hormone (TT4), total cholesterol (TC) and low density lipoprotein cholesterin (LDL-C). Rats in L-T-4 group were divided into normal lipid (NL) group) and high lipid (HL) group) according to lipid value of NC group. Monocytes were separated from blood to determine TLR4 expression by flow cytometry.

RESULTS: In NL and HL groups TSH were higher than in NC and Sham groups ($p<0.05$). TT4 have no significant differences ($p>0.05$). TLR4, TLR4 mRNA, NF-κB (p65) were increased ($p<0.05$). TNF-α, IL-6 and IL-1β were higher than in NC and sham groups ($p<0.01$). There were no significant differences of TLR4, TLR4 mRNA, NF-κB (p65), TNF-α, IL-6 and IL-1β expression between NL and HL groups ($p>0.05$).

CONCLUSION: TLR4, TLR4 mRNA, NF-κB (p65) of PBMC and TNF-α, IL-6, IL-1β expression in serum were all increased in SCH rats, which was not related to serum dyslipidemia.

INTRODUCTION

Subclinical hypothyroidism (SCH) is defined as an elevated serum thyroid-stimulating hormone (TSH) level associated with normal total or free T4 and T3 values. The morbidity of SCH in population is 4.6–9.5% (Osman *et al.* 1999; Lekakis *et al.* 1997) and it increases with aging (Osman *et al.* 1999). SCH often leads to the formation of early atherosclerotic plaque, thereby increases the incidence of coronary heart disease in clinical. Traditionally, serum dyslipidemia is thought to be the main reason of atherosclerosis formation (Ross 1999; Medzhitov *et al.* 1997; Bovijn *et al.* 2012). However, a recent large-scale clinical survey has reported that the difference of serum cholesterol level between normal people and SCH patients was not significant (Lekakis *et al.* 1997). SCH is a risk factor for atherosclerosis but not related with serum lipid level (SLL) (Hak *et al.* 2000).

Ross *et al.* have believed that beyond serum lipid level, inflammation exists in every stage from occurrence to formation of thrombus in atherosclerosis (Ross 1999). Toll-like receptors (TLR) are transmembrane protein located on cell membrane, which are called pathogen-associated molecular patterns (PAMPs), and they can recognize the conservative molecular composition of microorganism in certain types. Medzhitov *et al.* cloned and identified a homologue of the drosophila Toll protein and named it as TLR4 in 1997 (Medzhitov *et al.* 1997). The extracellular domain of TLR4 protein consists of more than 600 amino acids and the intracellular domain of TLR4 is TIR (Toll/IL-1 receptor), composed of 3 highly conserved regions and 150 amino acids. The TIR domain regulates the interaction between TLR and constitutive protein in signal transduction (Bovijn *et al.* 2012). TLR can recognize lipopolysaccharide, lipoteichoic acid, heat shock protein (HSP) and the extracellular domain A of fibronectin (EDA). TLR4 expresses in all kinds of cell lines and distributes widely in T lymphocytes, B lymphocytes, mononuclear macrophages, myocardial cells and epidermal microvascular cells. Activated TLR4 can induce the generation of cytokines and chemotactic factor (Guha & Mackman 2001). Monocytes play important roles in the development of atherosclerosis in blood circulation. In early atherosclerosis, activated endothelium expressed cell adhesion molecules, and triggered monocytes in the blood to roll along the surface of vascular and adhere to active point (Eriksson *et al.* 2001), and then run into plaques to differentiate into macrophages to be foam cell after swallowing lipid peroxide (Eriksson *et al.* 2001], and so atherosclerosis formed finally. There is plenty of evidence that TLR4 influences the occurrence of atherosclerosis through several pathways.

Recently it has reported that C-reactive protein value increases significantly in SCH patients (Gerlach *et al.* 2003), suggesting that inflammation maybe involved in the pathologic changes when SCH causes atherosclerosis. Anyway, whether SCH affects the TLR4 expression of monocyte in peripheral blood of rats has not been reported yet and the molecular mechanism is not clear. We supposed that the increased TSH in the blood of SCH rats will influence the expression of TLR4. We aim to detect the changes of lipid inflammatory cytokines tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6) and Interleukin-1β (IL-1β) in SCH rats blood and TLR4 expression in peripheral blood, we want to check that whether increased TSH in SCH rats will affect the TLR4 expression through the change of serum lipid level and explore the signal transduction mechanism that how increased TSH influences the TLR4 expression in peripheral blood.

MATERIAL AND METHODS

Establishment of SCH rat model

The ethics committee from Chinese national human genome center at shanghai approved all aspects of this study. Fifty wistar female rats (150–180 g, available from Shanghai Institute of Laboratory Animal Center), were fed with normal diet plus distilled water (NDPDW) for a week, then divided into three groups randomly; normal control group (NC group, n=10), Sham group (n=10) and L-thyroxine treated group (L-T-4 group, n=30). Rats in NC group were kept on feeding with NDPDW. Rats in sham group were anesthetized by 10% chloral hydrate. Skin of neck was prepared and made an incision to expose the thyroid gland, then sutured, rats were fed with NDPDW and weighed every two days. Rats in L-T-4 group were anesthetized by 10% chloral hydrate. Cervical skin was prepared and made an incision. After separating the connective tissue and sternohyoid, the thyroid gland was exposed and performed bilateral thyroidectomy and then the skin was sutured. The rats were fed with rich-calcium water (0.1% $CaHCO_3$) and normal food. Then, the weight was recorded every two days. At the 6th week of post-operation, rats in L-T-4 group were injected with L-thyroxine (L-T-4) 0.95 μg/100g/d (Sigma, America) subcutaneously for 3 weeks. In contrast, rats in sham group were given the same dose of placebo (PBS) and rats in NC group were kept on feeding with NDPDW. At the end of the 8th week, blood (2 mL) was collected from the tails and used to determine serum total cholesterol (TC), low density lipoprotein cholesterin (LDL-C), TSH and total thydroxine 4 (TT-4). The rest of blood was used to separate monocytes.

Determination of TC, LDL-C, TSH and TT-4 in serum

Blood was centrifuged at 3000 rpm for 20 min, then the serum was separated to determine TSH and TT-4 using immuno-chemiluminesence assay (ICMA) (DPC, USA). TC and LDL-C were determined using automatic biochemistry analyzer (Siemens Corp., Germany).

Separation of monocytes

Blood (20 mL) was centrifuged at 2000 rpm for 20 min to separate cells in the lower precipitation (10~12.5 mL). Hank's solution (without Ca^{2+}, Mg^{2+}, pH 7.2~7.6) of the same volume was added into the cells to get the suspension. Cell component was separated by Ficoll-Hypaque. Then, monocytes were collected from the interface of plasma layer and lymphocyte lipid. Hank's solution of 3–4 times volume was added into obtained monocytes and mixed gently by capillary pipet. The mixture was centrifuged at 1500 rpm for 10 min, and the supernatant was removed. Cells were washed using Hank's solution for 2 times and centrifuged at 1500 rpm for 10 min. Finally, the residual lymphocyte lipid was removed. Then, cells were further washed by RPMI-1640 medium and the supernatant was removed. Finally, the cells were suspended with Hank's solution containing 20% bovine serum (0.2 mL/mL) again and cultured in incubator with 5% CO_2 at 37 °C for 2–3 h. Then the supernatant was removed and the adherent monocytes were available.

Detection of TLR4 by flow cytometry

Monocytes were incubated in rabbit anti-mouse TLR4 antibody (Boster, China) diluted 1:100 at 4 °C overnight, and washed with sodium azide for three times. Finally incubated with goat anti-rabbit IgG (H+L) labeled with Fluorescein Isothiocyanate (FITC) (Santa Cruz, America) diluted 1:50 for 2 hours at 37 °C. At the same time, cells in NC group were incubated with mouse anti-human IgG_{2a} (eBio-science, America) diluted 1:50 for 2 h at 37 °C, then centrifuged at 13 000 rpm for 5 min at 4 °C and washed with sodium azide for three times, the supernatant was removed and the cells were diluted with sodium azide again, then the TLR4 expression was analyzed by flow cytometry.

Detection of TLR4 and NF-κB (p65) protein by western blot

Cells were lysed in a lysis buffer containing 2% sodium dodecyl sulfate (SDS) and 0.125 M Tris-HCl (pH 6.8) on ice for 20 minutes, then followed high-speed centrifugation, the supernatant protein was collected. SDS-PAGE was performed using 10% polyacrylamide gel. PAGE separated proteins were electrophoretically transferred onto nitrocellulose membranes (Canaris *et al.* 2000). The membrane filters were blocked with 10% fat-free milk in TBST (0.1% Tween 20) for 1 hour and then incubated with rabbit anti-mouse antibody (1:200; Boster, China), NF-κB (p65) monoclonal antibody (1: 1000; Santa Crutz, USA) and GAPDH diluted 1:1000 in TBST at 4 °C overnight, and finally incubated with HRP anti-rabbit secondary antibody (Santa Crutz, USA) or anti-mouse IgG (Invitrogen Life Technologies, Carlsbad, CA, USA) diluted 1:2000 for 1 hour at room temperature. The antigen-antibody complex on the membrane was detected with enhanced chemiluminescense detection reagents (Tiangen, biotechnology, Co., Ltd., China), and bands were determined using Leica image analysis software.

Detection of TLR4 mRNAs level by Real- time PCR

Total monocyte RNA was extracted with TRIzol (Invitrogen Life Technologies), and cDNA were synthesized from 1 μg RNA with Reverse Transcription kit (Invitrogen Life Technologies), which were both according to the manufacturer's protocol. The real-time quantitative PCR analyses were performed in triplicate using iQ™ SYBR® Green Supermix kit (Bio-Rad, USA). The β-actin gene was chosen as an endogenous control. The primers used for TRL4 were: forward, 5'-GCCGAAAGGTGATTGTTGTGGTGT-3' and` reverse, 5'-ACTGCCAGGTCTGAGCAATCTCAT-3'. The primers used for β-actin were: forward, 5'-ACCAACTGGGACGACATGGAGAAA-3' and reverse, 5'-TAGCACAGCCTGGATAGCAACGTA-3'. The 25 μL reaction (composed of 12.5 μL SYBR® Green supermix, 1 μL primers mixture, 0.5 μL cDNA and 11 μL anhydrous RNase) conditions were: 94 °C for 5 min, 95 °C for 1 min, 54 °C for 45 sec and 72 °C for 1 min, repeated for 35 cycles (for TLR4) or 30 cycles (for β-actin), and 10 minutes of extension at 72 °C. Data was analyzed by ABI Prism 7300 SDS Software.

Detection of Inflammatory mediators in supernatant by ELISA

The supernatant was thawed and centrifuged at 1 000 g for 10 min at 4 °C, then TNF-α (Jiamay biotechnology Co., Ltd., China), IL-1β (Jiamay biotechnology Co., Ltd., China) and IL-6 (Shenzhen Juying, biotechnology Co., Ltd., China) were determined respectively according to the instruction of ELISA kits.

Statistical analysis

Statistical analyses were performed using SPSS 10.0 software, multi-group comparison was made by analysis of variance. Data results were presented as mean ± standard deviation ($\bar{x}$±SD), p-values <0.05 were considered statistically significant.

RESULTS

Rat survival and serum TSH, TT4, TC, LDL-C levels

There were 10 rats in NC group, 9 rats in Sham group and 27 rats in L-T-4 group survived. However, the TSH and TT4 levels of 3 rats in L-T-4 group were within the values of NC group, suggesting that there were 3 rats fail to perform experiment in L-T-4 group, so we abandoned these 3 data. L-T-4 group were divided into normal lipid group (11 rats, NL group) and high lipid group (13 rats, HL group) according to their TC and LDL-C values compared to NC group. Additionally, there were no significant differences in TSH, TT4, TC, LDL-C levels between NC and Sham groups (p>0.05) (Table 1). TSH values were

Tab. 1. The levels of Serum TSH, TT4, TC and LDL-C in 4 groups.

		NC group (n=10)	Sham group (n=9)	NL group (n=11)	HL group (n=13)
	Survival rate (%)	100%	100%		
TSH (mIU/L)	$\bar{x}$±SD	0.071±0.022	0.072±0.021	0.394±0.029*	0.345±0.033*
	95% CI	0.016~0.239	0.018~0.236		
TT4 (μg/dL)	$\bar{x}$±SD	4.06±0.96	4.10±0.98	4.01±0.92	4.13±1.27
	95% CI	2.33~5.98	2.39~6.01		
TC (mmol/L)	$\bar{x}$±SD	3.20±0.71	3.21±0.77	3.16±0.74	5.01±0.65*
	95% CI	2.17~4.12	2.32~4.52		
LDL-C (mmol/L)	$\bar{x}$±SD	2.36±0.26	2.21±0.24	2.24±0.23	3.56±0.45*
	95% CI	2.01~3.79	1.87~3.70		

*p<0.05 *vs.* NC and Sham group. CI: confidence interval; NC: normal control group; NL: normal lipid group; HL: high lipid group; TSH: thyroid-stimulating hormone; TT4: total thyroid hormone; TC: total cholesterol; LDL-C: low density lipoprotein cholesterin.

significantly higher in NL and HL groups than those in NC and Sham groups (p<0.05), and no significant difference of TT-4 value was detected among these 4 groups, suggesting that SCH model was successfully established. TC and LDL-C values were significantly higher in HL group compared with those in NC and Sham groups (p<0.05).

The change of TLR4 expression in peripheral blood mononuclear cells (PBMC) of SCH rats

Mean fluorescence intensities (MFI) of NC, Sham, NL and HL group were 10.1±0.5, 9.7±0.6, 18.3±1.3 and 25.4±1.1/10^5 monocytes, separately (Figure 1). There was no significant difference between NC and Sham group (p>0.05). MFIs of NL and HL groups were significantly higher than those of NC and Sham group (p<0.05), but there was no statistical difference between them (p>0.05). So we can see that TLR4 expression in PBMC of SCH rats has a significant rise, not by virtue of the change of serum lipid level.

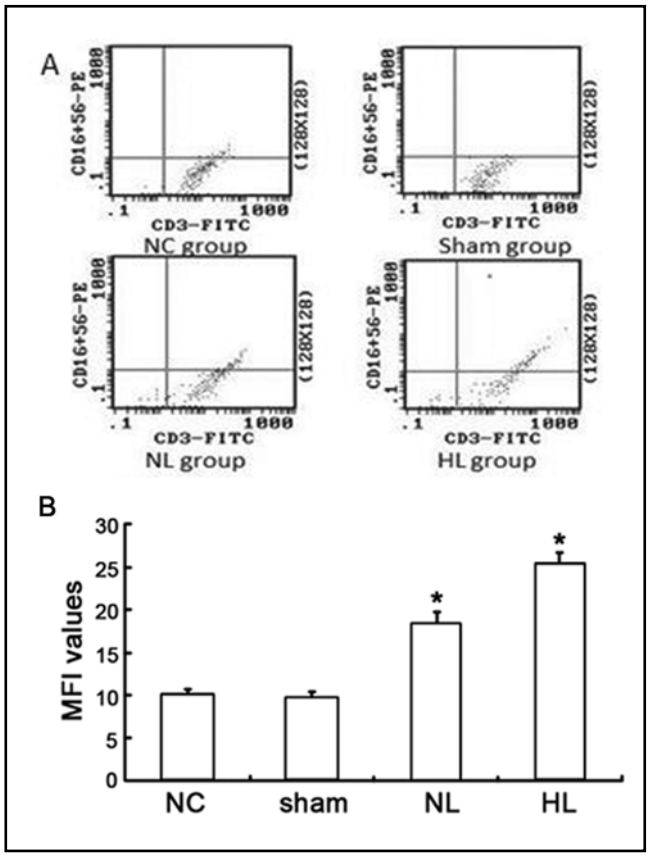

Fig. 1. The change of TLR4 expression in PBMC of SCH rats, incubated by anti-TLR4, and determined by flow cytometry. **A:** MFI values of TLR4 protein of monocytes. **B:** MFI values every per 10^5 monocytes. *p<0.05. *vs.* NC and Sham groups. NC: normal control group; NL: normal lipid group; HL: high lipid group.

The change of TLR4 protein expression in PBMC of SCH rats

The protein of TLR4 was detected by western blot. The ratios of TLR4/GAPDH in NC and Sham group were 0.241±0.010 and 0.230±0.008 respectively and there was no significant difference between them (p>0.05) (Figure 2). The values in NL and HL groups were 0.442±0.005, 0.558±0.009 respectively and the difference was not significant (p>0.05). Their values were significantly increased compared with NC group (p<0.05). All these results suggested that TLR4 expression in PBMC of SCH rats increased significantly, but it was irrespective of the rise of serum lipid (TC, LDL-C) levels.

The change of TLR4 mRNA expression in PBMC of SCH rats

The change of TLR4 mRNA expression was detected by RT-PCR (Figure 3). The relative expression levels of TLR4 mRNA in NC, Sham, NL and HL group were 1.00±0.13, 0.98±0.09, 2.61±1.10 and 3.44±0.45, respectively. There was no significant difference between NC and Sham group (p>0.05). TLR4 mRNA expression levels in NL and HL groups were significantly higher

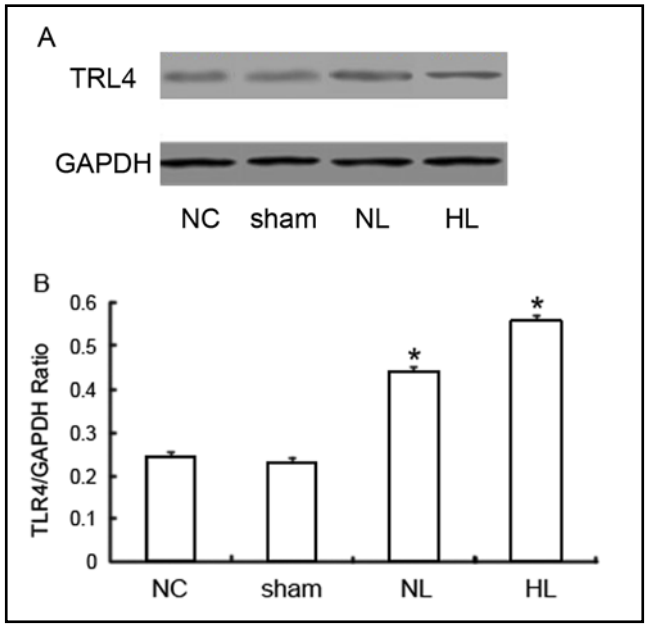

Fig. 2. The change of TLR4 protein expression in PBMC of SCH rats. **A:** Strips of TLR4 and GAPDH. **B:** TLR4 / GAPDH ratio by photodensitometry. *p<0.05 vs. normal control and Sham groups. NC: normal control group; NL: normal lipid group; HL: high lipid group.

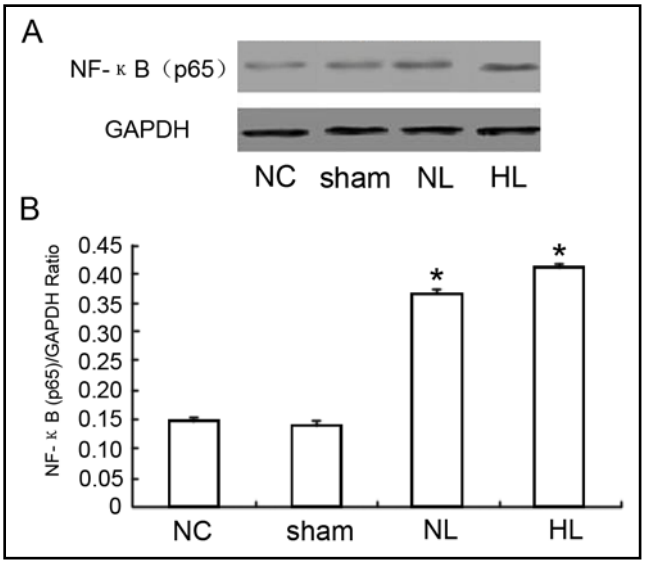

Fig. 4. The change of NF-κB p65 protein expression in PBMC of SCH rats determined by Western blot. **A:** The expression of NF-κB p65 and GAPDH. **B:** NF-κB p65/GAPDH ratio determined by photodensitometry. *p<0.05 vs. normal control and Sham groups. NC: normal control group; NL: normal lipid group; HL: high lipid group.

than those in NC and Sham group (p<0.05).This results suggested that TLR4 mRNA level was highly increased in PBMC of SCH rats. TLR4 mRNA expression level in HL group showed a higher level than that in NL group, but there was no significant difference (p>0.05), suggesting that the increased serum lipid (TC, LDL-C) level can promote the expression of TLR mRNA, however, there was no statistical difference between them.

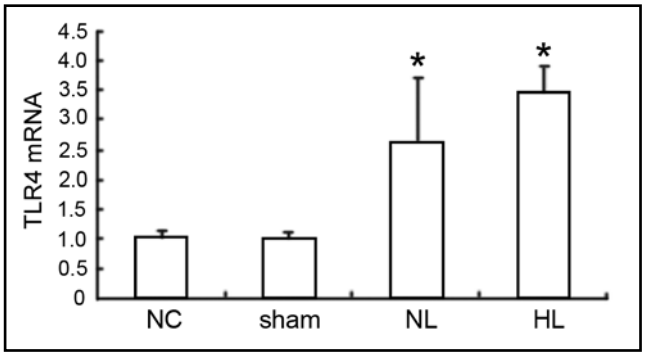

Fig. 3. The change of TLR4 mRNA expression in PBMC of SCH rats determined by RT-PCR. *p<0.05 vs. normal control group. NC: normal control group; NL: normal lipid group; HL: high lipid group.

The alteration of NF-κB(p65)protein expression in PBMC of SCH rats

This change was detected by western blot (Figure 4). The ratios of NF-κB (p65)/GAPDH in NC and Sham groups were 0.146±0.003 and 0.138±0.005, respectively. Those in NL and HL groups were 0.364±0.004 and 0.411±0.004, respectively. The ratios were significantly increased in HL and NL groups compared with those in NC and Sham groups (p<0.05). There was no significant difference between NC and Sham group (p>0.05), as well as between HL and NL groups. These results suggested that NF-κB (p65) level was significantly increased in PBMC of SCH rats. The increased serum lipid (TC, LDL-C) levels may promote the expression of NF-κB (p65), but there was no statistical difference between them.

The variation of monocyte with TNF-α, IL-6 and IL-1β in peripheral blood of SCH rats

Serum TNF-α, IL-6 and IL-1β were determined by ELISA (Figure 5). TNF-α values in NC, Sham, NL, and HL groups were 13.59±0.14, 13.48±0.12, 64.98±2.72 and 75.96±2.16 pg/ml, respectively. IL-6 values were 5.81±0.50, 5.79±0.45, 35.29±0.74 and 42.40±1.57 pg/ml. IL-1β values were 10.06±0.25, 9.98±0.24, 70.07±5.35 and 85.60±4.79 pg/ml. TNF-α, IL-6 and IL-1β levels significantly increased in NL and HL groups compared with those in NC and Sham groups (p<0.01). But there was no significant difference between NL and HL group (p>0.05).

DISCUSSION

Analysis of the reasons for the change of lipid level in SCH

The change of lipid level in SCH human and animals has always been controversial. Canaris *et al.* (2000) reported in 2002 that lipid TC levels of SCH rats increased with the rise of TSH level, and presented a linear relationship. Chen *et al.* had recently found

that blood TG, TC, LDL-C, APoB and Lp(a) levels of SCH patients were significantly higher than normal persons (Chen 2012). However, Bindels *et al.* (1999) and Pirich *et al.* (2000) had reported that there was no direct correction between SCH and lipid growth after a large-scale epidemiological survey. Iqbal *et al.* (2006) found that the serum LDL-C were significantly higher in 84 SCH subjects compared with 145 controls, and in the SCH females the TC levels were also significantly elevated. We have analyzed the reason of these inconsistencies: they are all clinical researches, with patients having different ages, gender and degrees of SCH (Staub *et al.* 1992), and all these can contribute to the inconsistency of result. Deng *et al.* reported in 2008 (Deng *et al.* 2008) that 37 of 60 SCH patients (62%) had increased lipid levels, and this was basically agreed with our research. Our work was a basic research and the rats with the same baseline conditions, intervening factors, rearing environment, feed and basically same sacrifice weight. However, it turned out that TC and LDL-C levels of 11 rats were within normal limits, 13 rats had significantly increased serum lipid, while TSH and TT4 levels had no significant difference. So we can conclude that the rise of TSH might be just one of the precipitating factors in the lipid change when SCH occurred.

The reason of TLR4 expression rise of monocytes in peripheral blood of SCH rats

Conventional view was that the rise of TSH in SCH caused the decrease of LDL receptors on the surface of liver, and then the synthesis of liver cholesterol reduced, the excretion of cholesterol and its metabolites from the bile became lower at the same time, so the concentrations of TC and LDL-C increased (Lee *et al.* 2011; Lai *et al.* 2011). We found that the change of serum lipid was not consistent but the results of activations to TLR in peripheral blood of SCH rats were identical (no significant TLR protein expression changes between NL and HL groups), suggesting that the activations to TLR4 was not from serum dyslipidemia.

We can see that the rise of TSH can induce the TLR expression in peripheral blood of SCH rats, and activate NF-κB (p65), and then increase mRNA and TLR4 protein expression. Further research is needed to elucidate that whether TSH activates TLR4 directly or whether TSH is the ligand of TLR4. Previous studies have reported that the artery endothelial dysfunction of SCH patients becomes worser when TSH level rises (Kahaly 2000). Therefore, we reckon that the endothelial cell damage induced by TSH can cause the production of cytokines which can activate the monocytes in blood, and then increase TLR4 expression. We can also conclude that the changes caused by TSH are not from serum dyslipidemia, in other words, the rise of lipid can't activate TLR.

The ELISA results indicated that inflammatory cytokines, such as TNF-α, IL-6 and IL-1β were significantly higher in peripheral blood of SCH rats, but the change was not caused by the lipid level (TC and LDL-C). As the reasons of cell inflammatory cytokines elevated, we considered that the activation of mononuclear cells was one of the reasons for this change. In previous study, we have found that the activated TLR4 can secrete these inflammatory cytokines (Yang *et al.* 2012) and TLR4 exist in vascular endothelial cells (Bulut *et al.* 2002), they also can secrete some cytokines after been activated. Whether the increased TSH in blood can activate TLR4 which existed in vascular endothelial cells, and induce the cytokines excretion are needed in further research.

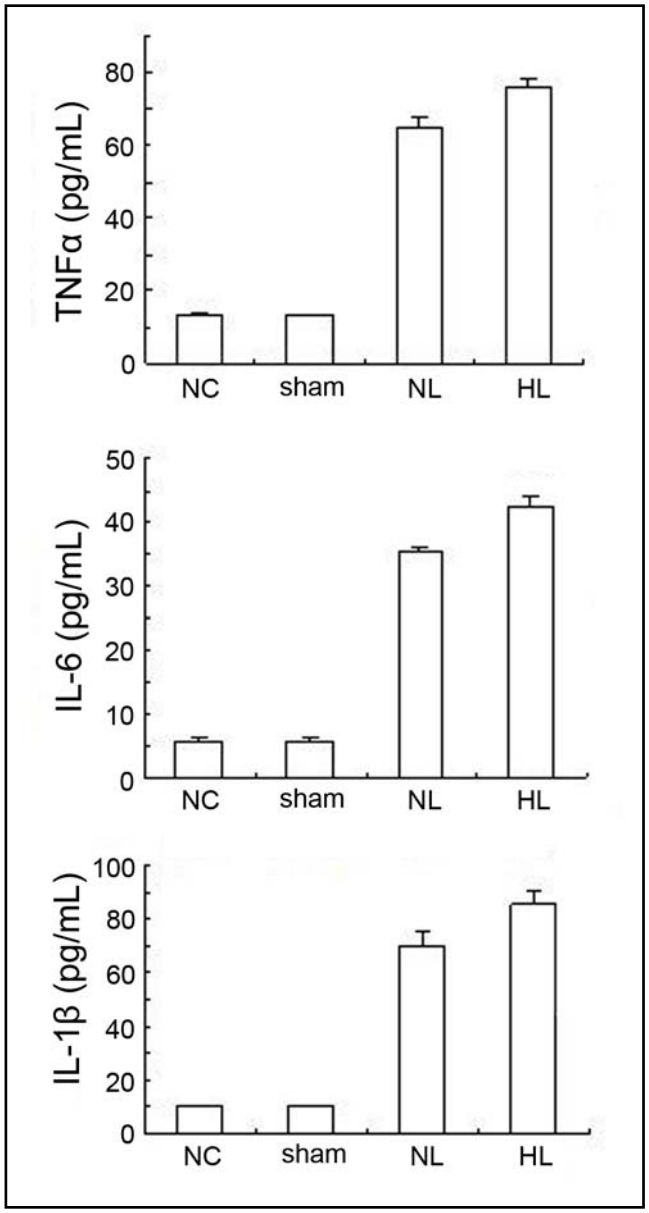

Fig. 5. The change of TNFα (**A**), IL-6 (**B**) and IL-1β (**C**) expression in peripheral blood of SCH rats determined by ELISA. **p*<0.05 *vs.* NC and Sham groups. NC: normal control group; NL: normal lipid group; HL: high lipid group.

CONCLUSION

Our study suggests that the rise of TSH in serum might induce the TLR expression and activate NF-κB (p65), and then increase TLR4 mRNA and protein expression in peripheral blood. Besides, the inflammatory cytokines, such as TNF-α, IL-6 and IL-1β increase in serum of SCH rats. The clinical significance of high-normal TLR4, NF-κB and inflammatory cytokines levels with regard to SCH prognosis should be determined in prospective studies, providing new ideas for the treatment of SCH.

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