Role of Glycogen Synthase Kinase3β/GATA-4 in Tumor Necrosis Factor-α-Regulated Sarcoplasmic Recticulum Ca^{2+}-ATPase Expressions in Cardiomyocytes

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Background: Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) plays an essential role in Ca^{2+} homeostasis and cardiac functions. Tumor necrosis factor-α (TNF-α) decreases the SERCA2a, which can induce cardiac dysfunction. GATA-4 is a critical transcription factor in load-mediated SERCA2a expression and negatively regulated by glycogen synthase kinase3β (GSK3β). The present study was to evaluate whether TNF-α can modulate SERCA2a via GSK3β and GATA-4.

Methods: To determine whether TNF-α could modulate the expression of SERCA2a, GSK3β, and GATA-4, HL-1 cells were treated with TNF-α (50 ng/ml) for 24 hours. The expressions of SERCA2a, GSK3β and nuclear GATA-4 were measured by real-time RT-PCR and immunoblot analysis.

Results: TNF-α decreased the RNA expression of SERCA2a by 30 ± 8%. In contrast, the protein level of GSK3β was not significantly changed by TNF-α. In addition, TNF-α did not alter the protein expression of nuclear GATA-4.

Conclusion: TNF-α decreases SERCA2a expression. These findings suggest that GSK3β and GATA-4 may not play an important role in SERCA2a modulation during acute inflammatory stage.

Key Words: G3K3β • GATA-4 • Heart failure • SERCA2a • Tumor necrosis factor-α

INTRODUCTION

The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a) plays an essential role in Ca^{2+} homeostasis and regulates cardiac functions. Reduction in the expression of SERCA2a has been widely documented in failing human heart and also in animal models of heart failure.[1,2] Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, plays a vital role in the pathogenesis of cardiovascular diseases, including heart failure, myocarditis, acute myocardial infarction and sepsis-related cardiac dysfunction.[3-8] TNF-α was elevated in patients with congestive heart failure and correlated with the severity of heart failure.[3] Moreover, TNF-α can down-regulate the RNA and protein level of SERCA2a,[9,10] which may contribute to cardiac dysfunction. TNF-α has been shown to decrease SERCA2a expressions through epigenetic regulation from promoter methylation.[11] However, the molecular mechanisms underlying the effects of TNF-α on the SERCA2a transcriptional regulation were not fully elucidated.

SERCA2a transcription is regulated in a complex manner through several constitutive and inducible transcription factors. GATA-4 is a critical transcription factor involved in survival of cardioblasts, terminal differ-

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entiation of cardiac myocytes,[12-14] and load-dependent SERCA2a promoter activity.[15] The transcriptional activity of GATA-4 is controlled by a nucleocytoplasmic shuttling mechanism, which is modulated by glycogen synthase kinase3β (GSK3β).[16] Direct phosphorylation of GATA-4 by GSK3β leads to its export from the nucleus and thus negatively regulates its transcriptional activity. However, it is not clear whether TNF-α may modulate SERCA2a expression through GSK3β or GATA-4. In this study, we evaluated the role of GSK3β and GATA-4 in TNF-α-regulated SERCA2a expression in a murine atrial cell line of HL-1 cardiomyocytes.

METHODS

Cell preparations

The HL-1 cells derived from mouse atrial cardiac muscle cells[17] (kindly provided by Dr. Claycomb) were cultured in a humidified atmosphere of 5% CO_2 at 37 °C in Claycomb medium (JRH Biosciences, Lenexa, KS) supplemented with penicillin-streptomycin, norepinephrine (GIBCO BRL) and 10% FBS (Sigma, St. Louis, MO). To determine whether TNF-α could modulate the expression of SERCA2a via the signaling pathway of GSK3β and GATA-4, we treated HL-1 cells with TNF-α (50 ng/ml, Sigma) for 24 h, and then harvested for further experiments.

RNA isolation and real-time RT-PCR

Total RNA was isolated from HL-1 cells and reverse transcribed by using the superscript III (Invitrogen, Carlsbad, CA). SERCA2a expression was analyzed by quantitative PCR with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI PRISM7300 thermocycler (Applied Biosystems). The primers used for SERCA2a forward and SERCA2a reverse were 5′-CGAGTTGAACCTTCCCACAA-3′ and 5′-CATCTGCCAGGACCATCTCA-3′, respectively. The primers used for GAPDH forward and GAPDH reverse were 5′-CGAGTTGAACCTTCCCACAA-3′ and 5′-CATCTGCCAGGACCATCTCA-3′, respectively. The relative changes in the transcript level of the SERCA2a were estimated from the threshold cycle (Ct) value and normalized to the respective Ct value of the GAPDH determined in the corresponding samples and subsequently to the control cells.

Nuclear protein isolation

After treated cells were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitor cocktails) and centrifugation, cell pellets were lysed by adding 25 μl of 10% Nonidet P-40 and followed by centrifugation at 12,000 *g* for 30 s. The pellets were resuspended in 50 μl of ice-cold buffer B (20 mM, HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktails) and centrifugation at 14,000 *g* for 5 min. The supernatant represented the nuclear fraction was stored at -80 °C for further experiments.

Immunoblot analysis

The protein content was determined by using Bradford analysis.[18] Equal amounts of total or nuclear proteins from the HL-1 cells were subjected to SDS-PAGE. Blots were probed with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) against GSK3β, GATA-4 and secondary antibodies conjugated with horseradish peroxidase. Bound antibodies were detected with the ECL detection system (Millipore) and analyzed with AlphaEaseFC software. Targeted bands were normalized to cardiac α-sarcomeric actin (Sigma) to confirm equal protein loading.

Statistical analysis

All quantitative data are expressed as the mean ± SEM. Unpaired Student *t*-test was used to compare the differences with or without incubation of TNF-α. A p-value lower than 0.05 was considered statistically significant.

RESULTS

TNF-α represses SERCA2a mRNA expression

To elucidate the possible role of TNF-α in the down-regulation of the SERCA2a expression, we examined the effects of TNF-α on the SERCA2a mRNA expression in HL-1cells. As shown in Figure 1, TNF-α (50 ng/ml) significantly decreased the SERCA2a mRNA level by 30 ± 8% as compared with control.

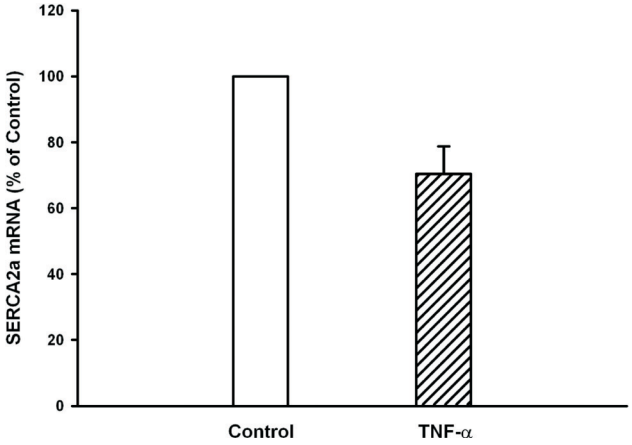

Figure 1. *Effects of TNF-α on SERCA2a mRNA level in HL-1 cells. The expression of SERCA2a mRNA was decreased by TNF-α (50 ng/ml, n = 4 independent experiments) through quantitative real-time PCR. The expression of the SERCA2a was normalized for GAPDH as internal control, and then normalized to the value of control cells. *p < 0.05 versus the control cells.*

Effects of TNF-α on protein level of GSK3β and GATA-4 in HL-1 cells

We investigated whether TNF-α could regulate SERCA2a expression through glycogen synthase kinase 3β (GSK3β) and GATA-4 signaling pathway. As shown in Figure 2, TNF-α treatment did not alter the protein level of GSK3β. TNF-α treatment also did not change the protein level of nuclear GATA-4 (Figure 3).

DISCUSSION

Previous studies have shown that TNF-α can regulate SERCA2a activity and proteins in cardiomyocytes,[9-11] which may contribute to the impaired heart function and arrhythmogenesis during heart failure or sepsis. However, knowledge about the molecular mechanism of TNF-α's effects on transcriptional regulation of the SERCA2a is still limited. Similar to that in the previous study, we found that 50 ng/ml of TNF-α decreased the RNA level of SERCA2a. These findings confirmed that TNF-α could modulate transcriptional level of SERCA2a. Failing human myocardium has been shown to be an abundant source of TNF-α production.[3,18-20] During sepsis, the serum TNF-α level may be drastically elevated and the intracardiac TNF-α level may be even significantly higher.[21-23] Therefore, the dosage of TNF-α used in this experiment is clinically relevant.

The regulation of the SERCA2a gene is intricate, involving physical and functional interaction between transcription factors and cofactors. In this study, we have found that TNF-α did not change the protein level of GSK3β and GATA-4 in HL-1 cells. Intra-nuclear GATA-4 has been identified as a critical factor in up-regulation expression of SERCA2a.[15] An increase in GSK3β may negatively regulate GATA-4 via phosphory-

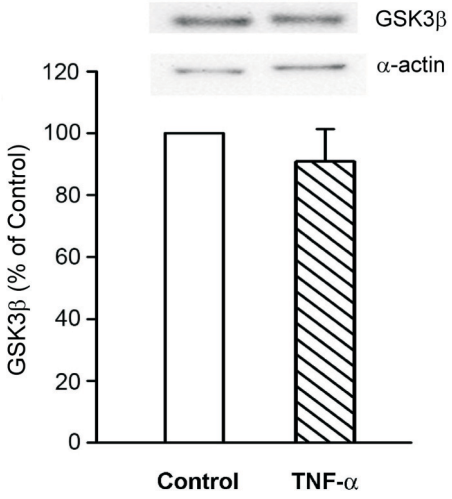

Figure 2. *Effects of TNF-α on GSK3β in HL-1 cardiomyocytes. Immunoblot analysis showed no significant changes of GSK3β expression by treating TNF-α (50 ng/ml, n = 3 independent experiments) for 24 hours in HL-1 cardiomyocytes.*

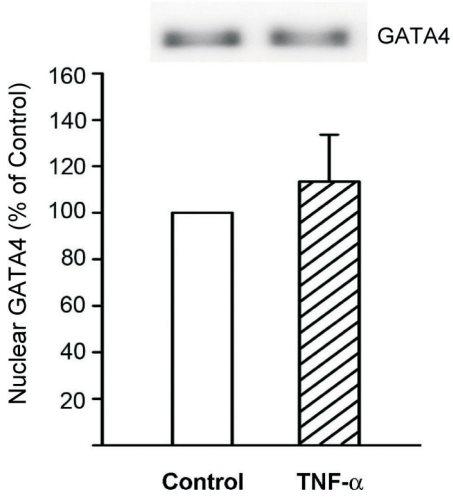

Figure 3. *Effects of TNF-α on GATA-4 in HL-1 cardiomyocytes. Immunoblot analysis show that nuclear GATA-4 level were no changed by treating TNF-α (50 ng/ml, n = 5 independent experiments) for 24 hours in HL-1 cardiomyocytes.*

lates GATA-4 to promote its export from the nucleus.[16] Our findings suggest that TNF-α did not regulate the SERCA2a though this pathway. However, it is not clear whether the phosphorylated GATA-4 in nuclei will be changed. Other signal pathways should be studied in the future to delineate the mechanism of TNF-α effect on SERCA2a expression.

Previous studies have shown that cardiac hypertrophy is linked to heart failure not only by enlargement of myocyte size but also by changing expressions of atrial natriuretic factor, and β-myosin heavy chain.[24-26] In addition, the apotosis and fibrosis of cardiac myocytes in heart failure are associated with GSK3β activity.[27] GATA-4 is a pivotal regulator and is possibly phosphorylated by GSK3β to inhibit its effects on gene expression in chronic heart failure.[28] However, we do not observe the involvement of GSK3β/GATA-4 in TNF-α-modulated changes of SERCA2a. In addition, GSK3β kinase activity can be regulated by phosphorylation status without changing the protein level.[29] Thus, the unchanged protein level of GSK3β did not completely exclude the possibility that TNF-α may inhibit GSK3β pathway by altering its phosphorylation status.

The data in this study should be interpreted with caution due to the potential limitations. Although equal amount of nuclear protein was used in this experiment, it would be more convincing if histone protein had been detected as an internal control to demonstrate an insignificant change of GATA-4 protein in TNF-α-treated HL-1 cells. In addition, we did not measure the intra-nuclear GAPDH to exclude the possibility of contamination of cytosolic proteins. Moreover, as the incubation time of TNF-α is relatively short as compared to the conditions in vivo, it remains uncertain whether GSK3β could not be activated in this acute inflammatory phase or pre-fibrotic phase, and it is also possible that ventricular myocytes may behave differently.

CONCLUSION

TNF-α decreased SERCA2a expression, which may not be mediated by GSK3β and GATA-4 in acute inflammatory stage.

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