Tumor Necrosis Factor- Downregulates the Voltage Gated Outward K⁺ Current in Cultured Neonatal Rat Cardiomyocytes

— A Possible Cause of Electrical Remodeling in Diseased Hearts —

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Background Inflammatory cytokines have been reported to contribute to the progression of cardiac remodeling in various heart diseases and a remarkable prolongation of the monophasic action potential duration and reductions in the expression of Kv4.2 and K⁺ channel-interacting protein-2 (KChIP-2) in a rat autoimmune myocarditis model have been documented. In this study, the effect of tumor necrosis factor- (TNF-) on cultured cardiomyocytes was evaluated, focusing on the change in the voltage-gated outward K⁺ current and expression of related molecules.

Methods and Results Cardiomyocytes isolated from 1-day-old Lewis rats were cultured for 72 h and treated with TNF- (50 ng/ml) for an additional 48 h. The myocytes treated with TNF- showed a 22% reduction in the peak K⁺ current, which consisted of a transient outward K⁺ current (I_{to}) and 1.4-fold enhancement of the cell-capacitance in comparison with the control. Among the cardiac ion channel related molecules evaluated in this study, Kv4.2 and KChIP-2 mRNA exhibited remarkable reductions (p<0.05).

Conclusions Treatment with TNF- induced reductions in I_{to} as well as cellular hypertrophy in neonatal cultured myocytes, which indicates that TNF- might play a role in promoting electrical remodeling of cardiomyocytes under inflammatory conditions. (Circ J 2006; 70: 605–609)

Key Words: Cardiac remodeling; Cardiomyocytes; Kv4.2; Neonatal rats; TNF-; Transient outward K⁺ current

Cardiac remodeling is understood to be a functional and structural alteration of the cardiac tissue in various heart diseases. In pathological situations, these alterations may result in some changes in the electrophysiological properties (ie, electrical remodeling) and may play a role in the development of clinical arrhythmias. We recently reported structural and electrical remodeling in a rat experimental autoimmune myocarditis (EAM) model, which was characterized by a remarkable prolongation of the action potential duration (APD) and increased ventricular arrhythmogenicity, as well as reduced expression of the transient outward K⁺ (I_{to})-related molecules, such as Kv4.2 and K⁺ channel-interacting protein-2 (KChIP-2).^{1,2} Among the various triggers for the cardiac remodeling in this model, we speculated that inflammatory cytokines might initially have an effect on the electrical properties because the remodeling was most prominent in the acute inflammatory phase. Although several investigators have reported the importance of tumor necrosis factor- (TNF-), a repre-

sentative proinflammatory cytokine, in altering Ca²⁺ handling, which may lead to cellular hypertrophy as well as toxicity of the myocardium,^{3–10} its effect on the cardiac K⁺ channels is still unknown. In the present study, we evaluated the effect of TNF- on the function and expression of these channels and their related molecules as well as on cellular hypertrophy in cultured neonatal rat cardiomyocytes.

Methods

Isolation and Culture of Neonatal Rat Myocytes

Ventricular myocytes were isolated from 1-day-old Lewis rat ventricles by 0.2% collagenase (Sigma, St Louis, MO, USA). The 8×10⁵ myocytes were seeded onto fibronectin-coated 35-mm dishes and cultured for 72 h with a daily renewal of F-12 medium (F-12 mixture 10.62 g/L, HEPES 15 mmol/L, NaHCO₃ 22 mmol/L) containing transferring–insulin–selenite (5 µg/ml, each) and 10% fetal bovine serum. The myocytes were cultured for an additional 48 h with or without TNF-. For patch-clamp recordings, 2×10⁵ cells were seeded onto the same type dishes and cultured in the same manner. The viability of cultured myocytes was analyzed by MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay after the 48-h treatment with TNF-. Trypan blue staining was also performed and the number of viable myocytes was counted

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Table 1 Absolute Copy Numbers of the mRNA/ 15 ng Total RNA in Myocytes After 48-h Treatment With 50 ng/ml of TNF-

Gene		Control	TNF-
Kv4.2	$\times 10^3$	5.18 $\pm$ 1.61	0.77 $\pm$ 0.15*
Kv1.5	$\times 10^4$	6.05 $\pm$ 4.10	2.98 $\pm$ 0.43
Kv4.3	$\times 10^3$	2.15 $\pm$ 0.47	1.13 $\pm$ 0.65
Kv1.4	$\times 10^3$	13.45 $\pm$ 5.77	5.61 $\pm$ 3.12
Erg	$\times 10^4$	4.53 $\pm$ 2.43	3.79 $\pm$ 0.11
Frequeenin	$\times 10^3$	8.27 $\pm$ 0.73	17.55 $\pm$ 2.87
KChIP2	$\times 10^3$	15.23 $\pm$ 4.42	5.14 $\pm$ 2.26*
SERCA2a	$\times 10^5$	4.95 $\pm$ 2.37	3.43 $\pm$ 0.81
RyR	$\times 10^4$	4.31 $\pm$ 0.13	8.12 $\pm$ 2.65
NCX	$\times 10^4$	1.61 $\pm$ 0.13	1.83 $\pm$ 0.44
L-Ca ²⁺	$\times 10^5$	8.06 $\pm$ 4.80	2.70 $\pm$ 3.21
Cyclophilin	$\times 10^5$	5.01 $\pm$ 2.40	13.52 $\pm$ 11.71*
GAPDH	$\times 10^6$	1.69 $\pm$ 0.09	3.60 $\pm$ 1.70*

* $p < 0.05$ vs control ($n = 5$ in each group).

after 24 and 48 h of treatment with TNF-. These protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Ethics Committee of the Kitasato University School of Medicine.

Electrophysiological Studies and Evaluation of Cell Size

Whole-cell patch-clamp recordings were performed at 37°C with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA) after culturing for 48 h with 50 ng/ml of TNF- and then comparison with the control. The normal Tyrode solution contained (in mmol/L) NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.5, NaH₂PO₄ 0.33, glucose 5.5, and HEPES 5 (pH 7.40 with NaOH). The pipette solution contained (in mmol/L) KCl 130, Na₂PO₄ 10, CaCl₂ 0.2, EGTA 0.1, MgATP 5, and HEPES 10 (pH 7.40 with HCl). While measuring the voltage-gated outward K⁺ currents, CdCl₂ (0.3 mmol/L) was added to normal Tyrode solution to inhibit L-type Ca²⁺ channels. The resistance of the pipette ranged from 2 to 6 M Ω . Outward K⁺ currents were obtained by applying 500 ms depolarizing pulses from a holding potential of -60 mV to various potentials between -30 mV to +50 mV in 10-mV steps. The initial peak current was identified as transient outward K⁺ current (I_{to}), because it was recorded in the presence of CdCl₂ (0.3 mmol/L), and thus was Ca²⁺-independent.¹¹ The current that was not inactivated at the end of the depolarization pulse was referred to as the steady-state outward K⁺ current (I_{ss}).¹¹ The cell capacitance (C_m) was calculated as the integrated area under the capacitive transient evoked by applying 10-mV depolarizing pulses from a holding potential of -60 mV. The cell capacitance and series resistance were electrically compensated by ~60–70%. The current signal was digitized and stored on a hard drive of an online computer by means of a DigiData interface and pCLAMP software (version 8.0; Axon Instruments). The sampling frequency was 2 kHz throughout the experiments.

The size of the cultured myocytes was evaluated by 2 methods. First, the C_m was measured as described above. Second, the microscopic 2-dimensional area in the NIH image was measured. The surface areas of 100 cells were traced in each culture and the mean was calculated.

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Western Blot Analysis

Quantitative real-time RT-PCR was performed as previously described.² cDNA was synthesized from 3 μ g of the total RNA purified from each sample and the mRNA levels of the voltage dependent K⁺ channels (Kv1.4, 4.2, 4.3, 1.5 and erg), I_{to}-regulating molecules (K⁺ channel-interacting protein-2; KChIP-2 and neuronal calcium center protein-1; frequenin), cardiac ion transporters (sarcoplasmic reticulum Ca²⁺-ATPase 2a; SERCA2a, ryanodine receptor; RyR and Na⁺-Ca²⁺ exchanger; NCX), L-type Ca²⁺ channels, and housekeeping genes (cyclophilin and GAPDH) were evaluated. The assay was performed with a QuantiTectTM SYBR Green PCR Master Mix (Qiagen, CA, USA) and primer pairs (2). An ABI PRISM 7700 System calculated a standard curve, and the absolute copy number of 13 species of the mRNA in the samples was obtained.

The level of the Kv4.2 protein in the myocytes was measured by Western blot analysis with an anti-Kv4.2 antibody as previously described.² Briefly, 50 μ g of a homogenized cardiac tissue with a lysis buffer containing 50 mmol/L of Tris-HCl (pH 7.4), 50 mmol/L of EDTA, 150 mmol/L of NaCl, 0.1% Triton-100, 10 μ g/ml of aprotinin, 200 mmol/L of PMSF, and 1 mg/ml of leupeptin was separated by 10% SDS-polyacrylamide gel electrophoresis and electroblotted to a PVDF transfer membrane (Amersham pharmacia biotech, UK). The membrane was incubated with anti-Kv4.2 (CHEMICON International) and subsequently with the second antibody (goat anti-rabbit IgG antibody) conjugated to alkaline phosphatase, and then the band was developed. In order to confirm the quality of the samples, 50 μ g of those were electrophoresed in the same manner, and the gel was stained with 0.25% CBB-R250. The bands near 37 kDa were used as the internal controls.

The densities of each band in the digitized images were measured using the public domain NIH image program. The density of Kv4.2 band was corrected with that of the internal control band.

Statistical Analysis

All quantitative data are described as mean $\pm$ SEM. Basic comparative statistics were performed using an unpaired Student's t-test. The quantitative RT-PCR data in Table 1 and the data from the Western blot analysis in Fig 3B were analyzed by a nonparametric test. The time-course of Kv4.2 mRNA expression in Fig 3A was analyzed by a repeated measure Friedman's test (The Numerical Algorithms Group Ltd). In the statistical analysis, $p < 0.05$ was considered to be statistically significant.

Results

Cell Viability and Size

There was no significant difference in viability analyzed by the MTT assay and trypan blue stain among myocytes treated with 0, 10 and 50 ng/ml of TNF- (data not shown). We performed the following experiments with 50 ng/ml of TNF- because the concentration of TNF- has been reported to be higher than 15 ng/g in cardiac tissue in EAM rats.^{12,13}

The 2-dimensional surface area and cell capacitance of cardiomyocytes were significantly larger in the TNF-group than in the control (Fig 1).

Fig 1. Effect of the tumor necrosis factor- (TNF-) on (A) the 2-dimensional cell-surface area and (B) cell capacitance of neonatal rat cardiomyocytes in the control and TNF- treated groups. In both evaluations, the cell sizes were significantly increased in the TNF- group. See text for details.

Fig 2. Effect of tumor necrosis factor- (TNF-) on the outward K^+ currents. (Panel A) Representative current trace in the control and TNF- treated cultured myocytes (Left and Right panels, respectively) obtained with depolarizing pulses with a 500 ms duration from a holding potential of -60 mV . The peak and late currents were reduced in the TNF- group in comparison with the control. (Panel B) Current-voltage relationship of the peak and late currents. TNF- significantly reduced both components of the outward K^+ currents. See text for details. * $p < 0.05$, ** $p < 0.01$ vs control.

Outward K^+ Current

Fig 2A shows representative current traces of the outward K^+ currents in the control and TNF- treated cultured myocytes. These currents were significantly reduced in TNF- treated myocytes compared with the control. Fig 2B shows the current-voltage relationships of both peak and late currents. The peak current (I_{to}) was decreased in the TNF- group ($3.18 \pm 0.13\text{ pA/pF}$) compared with the control ($14.77 \pm 3.88\text{ pA/pF}$) at 50 mV . The late current (I_{ss}) was also lower in the TNF- group ($2.50 \pm 0.30\text{ pA/pF}$) than in the control ($6.83 \pm 1.42\text{ pA/pF}$) at 50 mV .

Expression of Related Molecules

The absolute copy numbers of the 13 species of mRNA are shown in Table 1. Among those studied, Kv4.2 and KChIP-2 exhibited a significant reduction with treatment

with 50 ng/ml of TNF- . The expression of Kv1.5 and Kv1.4 tended to be decreased in comparison with the increased expression of the housekeeping genes (GAPDH and cyclophilin). The molecules related to Ca^{2+} -handling, such as ryanodine receptor, the L-type Ca^{2+} channel, SERCA and NCX , did not show any specific changes. Fig 3A shows the time course of Kv4.2 mRNA expression in the 2 groups. Kv4.2 mRNA expression in the TNF- group was significantly reduced in a time dependent manner (Fig 3A). The expression level of Kv4.2 protein was also reduced in the TNF- group (Fig 3B).

Discussion

This study evaluating the effect of TNF- on the voltage dependent outward K^+ current and related molecules in

Fig3. Effect of tumor necrosis factor- α (TNF- α) on the Kv4.2 expression. (Panel A) Time course of the Kv4.2 mRNA expression. Kv4.2 mRNA expression was reduced in a time-dependent manner in the myocytes in the TNF- α group ($p=0.03$ by a repeated measure non-parametric Friedman's test). (Panel B) Kv4.2 protein expression after a 48-h treatment with 50 ng/ml of TNF- α . The densities of the Kv4.2 bands corrected by the internal controls were also reduced in the TNF- α group ($p=0.04$ by a non-parametric test). See text for details.

cultured neonatal cardiomyocytes has several interesting findings. First, treatment with 10, 50 and 100 ng/ml of TNF- α did not have any significant influence on cell viability. Second, treatment with 50 ng/ml of TNF- α caused a significant increase in cell size and cell capacitance. Third, the total outward K^+ current was significantly reduced by TNF- α treatment. Finally, the expression levels of both mRNA and Kv4.2 protein were downregulated by TNF- α treatment. There are previous reports that have documented the effects of TNF- α on the various cardiac ion channels.¹⁴⁻¹⁷ Those reports support the dual effect of TNF- α on cardiomyocytes in different types or phases of cardiac injury. Our study shows that the TNF- α -induced reduction of the voltage gated K^+ channel may be a key mechanism of cardiac remodeling in rat autoimmune myocarditis.

Effect of TNF- α on the Cardiac K^+ Current

It has been reported that the level of TNF- α is increased in various heart diseases, especially myocarditis.^{3,4,12,13} Several reports have documented that TNF- α causes cellular hypertrophy (ie, structural remodeling)⁵⁻⁷ but we also speculated that TNF- α might cause some changes in the cardiac ion channels (ie, electrical remodeling), because we documented drastic changes in the APD as the result of a reduction in Kv4.2 expression in an acute myocarditis model (EAM).^{1,2} According to the present results, TNF- α promotes a reduction in the outward K^+ currents as well as cellular hypertrophy, at least in primary cultures of neonatal ventricular myocytes. TNF- α is considered to have an effect on the cardiac action potential through its effect on the I_{to} current.

Mechanism and Role of the Effect of TNF- α on the K^+ Currents

The mechanism of the effect of TNF- α on the cardiac K^+ currents is still unknown. In rat ventricles, I_{to} and some components of the delayed activated currents, such as I_{Kr} and I_{ss} , have been considered to generate Ca^{2+} -independent and depolarization activated outward K^+ currents. I_{to} is composed of at least 2 components; one is characterized by a fast recovery from inactivation ($I_{to,f}$) and another is a relatively slow recovery from inactivation ($I_{to,s}$). In rat ven-

tricles $I_{to,f}$ is encoded by Kv4.2 and Kv4.3, whereas $I_{to,s}$ is encoded by Kv1.4.¹¹ In the present study, the TNF- α -induced reduction of both Kv4.2 and KChIP2 could cause a decrease in $I_{to,f}$, which may result in a prolongation of the APD. These findings are in agreement with those in the ventricles of the rat EAM model. I_{ss} is considered to be encoded by Kv1.5 in the rat ventricle; however, the components of the delayed activated K^+ currents and the molecular basis have not been established. In the present study, there was a discrepancy between the changes in I_{ss} and Kv1.5 expression and further study is necessary to reveal the mechanisms of the reduction of I_{ss} .

Several reports document the regulatory signal transduction pathways of the K^+ channels gene expression.^{14,15} TNF- α modulates cardiac function through a number of signaling pathways, including cross-talk to the renin-angiotensin pathway or α -adrenergic pathway, and it also induces cardiac hypertrophy.^{5-7,17-24} Although the precise mechanisms of TNF- α -induced regulation of the K^+ channels are unknown, the cellular hypertrophy and subsequent intracellular Ca^{2+} -overload might modulate the K^+ channels' gene expression by activating the calcineurin/NFATc3 pathway.¹⁴ On the other hand, the change in the ion channel is not necessarily an outcome but may be also a cause of cellular hypertrophy. According to recent reports, a primary reduction in the I_{to} density could lead to myocardial hypertrophy and injury, probably because of the increased Ca^{2+} transient as a result of prolonged APD.²⁵⁻²⁷ Therefore, TNF- α may promote a worsening loop between structural and electrical remodeling (ie, cellular hypertrophy and I_{to} reduction in diseased hearts).

Study Limitations

First, because the myocytes were sampled from neonatal rats, the properties of the ion channels and cellular structures may differ from those of adult rat myocytes. Second, because the myocytes were cultured for 5 days under controlled conditions, the findings might differ from those for fresh myocytes. Therefore, the changes observed in this study may include a phenomenon under a modified environment.

Conclusion

TNF- caused a reduction in the outward K⁺ current and Kv4.2 expression, as well as cellular hypertrophy, in rat neonatal cultured myocytes. TNF- is considered to have an influence on electrical and structural remodeling and might produce an arrhythmogenic background in diseased myocardium.

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