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Tumor necrosis factor-α alters calcium handling and increases arrhythmogenesis of pulmonary vein cardiomyocytes

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Abstract

Inflammation and abnormal calcium homeostasis play important roles in atrial fibrillation. Tumor necrosis factor-α (TNFα), a proinflammatory cytokine, can induce cardiac arrhythmias. Pulmonary veins (PVs) are critical in initiating paroxysmal atrial fibrillation. This study was designed to investigate whether TNFα may change the calcium handling and arrhythmogenic activity of PV cardiomyocytes. We used whole-cell patch clamp and indo-1 fluorimetric ratio technique to investigate the action potentials, ionic currents and intracellular calcium in isolated rabbit single PV cardiomyocytes with and without (control) incubation with TNFα (25 ng/ml) for 7–10 h. The expression of sarcoplasmic reticulum ATPase in the control and TNFα-treated PV cardiomyocytes was evaluated by confocal micrographs and Western blot. We found that the spontaneous beating rates were similar between the control ($n=45$) and TNFα-treated ($n=28$) PV cardiomyocytes. Compared with the control PV cardiomyocytes, the TNFα-treated PV cardiomyocytes had significantly a larger amplitude of the delayed afterdepolarizations (6.0±1.7 vs. 2.6±0.8 mV, $P<0.05$), smaller L-type calcium currents, larger transient inward currents, larger Na^+–Ca^{2+} exchanger currents, a smaller intracellular calcium transient, smaller sarcoplasmic reticulum calcium content, larger diastolic intracellular calcium, a longer decay portion of the calcium transient (Tau), and a decreased sarcoplasmic reticulum ATPase expression. In conclusion, TNFα can increase the PV arrhythmogenicity and induce an abnormal calcium homeostasis, thereby causing inflammation-related atrial fibrillation.

Keywords: Atrial fibrillation; Calcium transient; Pulmonary vein; Tumor necrosis factor-α

Introduction

Atrial fibrillation is the most common sustained arrhythmia in clinical medicine and induces cardiac dysfunction and stroke. Inflammation is a well-known precipitating factor to cause atrial fibrillation (Chung et al., 2001; Seguin et al., 2004). But the pathophysiology of inflammation-induced atrial fibrillation is not clear. Tumor necrosis factor-α (TNFα), is an inflammatory cytokine that has been implicated in the pathogenesis of cardiovascular diseases, including heart failure, myocarditis, acute myocardial infarction and sepsis-related cardiac dysfunction (Levine et al., 1990; Low-Friedrich et al., 1992; Yokoyama et al., 1993; Latini et al., 1994; Packer, 1995; Kumar et al., 1996). TNFα also has been shown to be related to the occurrence of cardiac arrhythmias. Mice with a higher TNFα expression have a greater incidence of atrial and ventricular arrhythmias (London et al., 2003). Administrating TNFα in rats can induce arrhythmias with a loss of the myocyte inotropy (Krown et al., 1995). Previous studies have shown that TNFα

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changes the L-type calcium currents ($I_{Ca\text{-}L}$), and calcium transient in ventricular myocytes (Krown et al., 1995; Goldhaber et al., 1996; Cailleret et al., 2004). However, knowledge is limited about the effects of TNFα on the activity of the pacemaker cardiomyocytes, calcium regulation, Na^+–Ca^{2+} exchanger (NCX), and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a).

Pulmonary veins (PVs) are important foci of ectopic beats to initiate paroxysmal atrial fibrillation and ectopic atrial tachycardia (Walsh et al., 1992; Haissaguerre et al., 1998; Chen et al., 1999). Isolated PV specimens have been found to contain a mixture of pacemaker cells and working myocardium that have been suggested to be subsidiary pacemakers and cause atrial arrhythmias (Cheung, 1981; Masani, 1986; Blom et al., 1999; Perez-Lugones et al., 2003). The enhancement of the automaticity and triggered activity in the PV pacemaker cardiomyocytes contribute to the high PV arrhythmogenic activity (Chen et al., 2000, 2001, 2002a,b). Moreover, abnormal calcium handling may induce PV arrhythmogenesis (Honjo et al., 2003; Patterson et al., 2005). Both T-type and L-type calcium currents ($I_{Ca\text{-}L}$) play a role in causing PV arrhythmogenesis (Chen et al., 2002a,b, 2004). Additionally, modulating the NCX can regulate the PV electrical activity (Patterson et al., 2005; Wongcharoen et al., 2006). Because PVs have a critical role in the occurrence of atrial fibrillation, we hypothesize that TNFα may alter the calcium homeostasis and thereby increase PV arrhythmogenesis to initiate atrial fibrillation during inflammation or heart failure. Therefore, the purpose of this study was to investigate the effects of TNFα on the electrical activity, ionic currents, and calcium handling in the PV pacemaker cardiomyocytes.

Materials and methods

Isolation of PV cardiomyocytes

The investigation conformed to the institutional *Guide for the Care and Use of Laboratory Animals*. Male rabbits (n=89, weight, 1–2 kg) were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). The PV cardiomyocytes were enzymatically dissociated through the same procedure described previously (Chen et al., 2002a,b). Only cells with spontaneous activity and cross striations were used. The PV cardiomyocytes with pacemaker activity were identified by the presence of constant spontaneous beating. The experiments were performed in the PV cardiomyocytes incubated with normal Tyrode's solution (control) or with TNFα (25 ng/ml) for 7–10 h. The experimental time was determined according to the findings that the action potentials (APs) of PV cardiomyocytes with a short period (less than 5 h) of incubation with of the TNFα did not change significantly. The concentration of TNFα chosen in this experiment was known to have effects on cardiomyocytes in a previous study (Cailleret et al., 2004). The cells were allowed to stabilize in the bath for at least 30 min before the experiments.

Electrophysiological and pharmacological study

A whole-cell patch-clamp was performed using an Axopatch 1D amplifier at 35±1 °C, and borosilicate glass electrodes (o.d., 1.8 mm) were used, with tip resistances of 3–5 MΩ. Before the formation of the membrane-pipette seal, the tip potentials were zeroed in Tyrode's solution (containing in mM: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 10 and glucose 11; the pH was adjusted to 7.4 by titration with 1 N NaOH). The AP and membrane currents of the TNFα-treated and control PV cardiomyocytes were measured during superfusion with normal Tyrode's solution with or without TNFα, respectively. As described previously (Chen et al., 2002a,b), the APs and ionic currents were recorded in the current-clamp mode and in the voltage-clamp mode, respectively. The AP duration (elicited by 2 Hz electrical stimulation) at 20% (APD_{20}), 50% (APD_{50}) and 90% (APD_{90}) of the full repolarization was only measured in the PV cardiomyocytes with a spontaneous beating rate of less than 2 Hz. The micropipettes were filled with a solution containing (in mM) CsCl 130, $MgCl_2$ 1, Mg_2ATP 5, HEPES 10, EGTA 10, NaGTP 0.1, and Na_2phosphocreatine 5, titrated to a pH of 7.2 with CsOH for the $I_{Ca\text{-}L}$ experiments, containing (in mM) NaCl 20, CsCl 110, $MgCl_2$ 0.4, $CaCl_2$ 1.75, tetraethylammonium 20, BAPTA 5, glucose 5, Mg_2ATP 5, and HEPES 10, titrated to a pH of 7.25 for the NCX current experiments, and containing (in mM) KCl 20, K aspartate 110, $MgCl_2$ 1, Mg_2ATP 5, HEPES 10, EGTA 0.5, LiGTP 0.1, and Na_2phosphocreatine 5, titrated to a pH of 7.2 with KOH for the potassium currents, transient inward currents, and AP experiments. The voltage command pulses were generated by a 12-bit digital-to-analog converter controlled by pCLAMP software (Axon Instruments). The recordings were low pass-filtered at half the sampling frequency. Data were sampled at rates varying from 2 to 25 kHz.

The $I_{Ca\text{-}L}$ were measured as an inward current during depolarization from −40 to +60 mV in 10-mV steps for 300 ms at a frequency of 0.1 Hz. The NaCl and KCl in the external solution were replaced by tetraethylammonium chloride and CsCl, respectively.

The transient outward currents (I_{to}) were studied with a double-pulse protocol. A 30-ms pre-pulse from −80 to −40 mV was used to inactivate the sodium channels, followed by a 300 ms test pulse to +60 mV in 10 mV steps at a frequency of 0.1 Hz. $CdCl_2$ (200 μM) was added to the bath solution to inhibit the $I_{Ca\text{-}L}$. The I_{to} were measured as the difference between the peak outward current and steady state current. The delayed rectified outward potassium currents (I_K) were measured from the peak outward current at the end of 1-s depolarization from −40 to +60 mV in 10 mV steps at a frequency of 0.1 Hz during the infusion of 200 μM of $CdCl_2$ and 2 mM of 4-aminopyridine in the bath solution to inhibit the $I_{Ca\text{-}L}$ and I_{to}.

The transient inward current was induced during depolarization from a holding potential of −40 mV to +40 mV for a duration of 3 s and the current was measured on the return to −40 mV. The amplitude of the transient inward current was measured as the difference between the peak of the transient current and mean of the current just before and after the transient current (Chen et al., 2002a,b).

Hyperpolarization-activated membrane currents were activated from −40 mV to test potentials ranging from −20 to −120 mV in 10 mV steps for 1 s at a frequency of 0.1 Hz under an infusion of 200 μM of $CdCl_2$ and 2 mM of 4-aminopyridine in the bath

solution. The amplitudes of the inward rectified K^+ current (I_{K1}) were measured as 1 mM barium sensitive currents.

The NCX current was elicited by depolarizing pulses between −100 to +100 mV from a holding potential of −40 mV for 300 ms at a frequency of 0.1 Hz. The amplitudes of the NCX currents were measured as 10 mM nickel-sensitive currents (Wongcharoen et al., 2006). The external solution (in mM) consisted of NaCl 140, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5 and glucose 10 with the pH adjusted to 7.4 and contained strophanthidin 10 μM (to block Na^+/K^+ pump), nitrendipine 10 μM (dihydropyrdine antagonist) and niflumic acid 100 μM (to block Ca^{2+} activated Cl^- currents).

Measurement of intracellular calcium

The intracellular calcium ($[Ca^{2+}]_i$) was recorded by a fluorimetric ratio technique through the same procedure as previously described (Wongcharoen et al., 2006). The fluorescent indicator indo-1 was loaded by incubating the myocytes at room temperature for 20 to 30 min with 25 μM indo-1/AM (Sigma). The PV cardiomyocytes were then perfused with normal Tyrode's solution at 35±1 °C for at least 20 min to wash out the extracellular indicator and to allow for intracellular deesterification of the indo-1. The background and cell autofluorescence were cancelled out by zeroing the output of the photomultiplier tubes using cells without indo-1 loading. We did the experiments for the control and TNFα-treated PV cardiomyocytes during superfusion with normal Tyrode's solution with or without TNFα at 35±1 °C, respectively.

Ultraviolet light of 360 nm with a monochromator was used for the excitation of the indo-1 from a xenon arc lamp which was controlled by a microfluorometry system (OSP100-CA, Olympus). The excitation light beam was directed into an inverted microscope (IX-70: Olympus). The emitted fluorescence signals from the indo-1/AM loaded myocytes were digitized at 200 Hz. We recorded the ratio of fluorescence emission at 410 nm and 485 nm. An $R_{410/485}$ was used as the index of the $[Ca^{2+}]_i$. The ratios of the $[Ca^{2+}]_i$ transient, peak systolic $[Ca^{2+}]_i$, diastolic $[Ca^{2+}]_i$, and decay portions of the ratio of the $[Ca^{2+}]_i$ transient (τ_{Ca}) were measured during a 1.5 Hz field-stimulation with 10-ms square-wave pulses at twice the diastolic threshold strength. The τ_{Ca} was determined by a monoexponential least-square fit (Wongcharoen et al., 2006). The sarcoplasmic reticulum Ca^{2+} content was estimated by adding 20 mM of caffeine after electric stimulation at 1.5 Hz for at least 30 s. The total sarcoplasmic reticulum Ca^{2+} content was measured from the peak amplitude of the caffeine-induced $[Ca^{2+}]_i$ transients.

Immunolabeling of the expression of sarcoplasmic reticulum Ca^{2+}-ATPase

The isolated control and TNFα-treated cells were fixed with 2% paraformaldehyde (pH 7.2) for 10 minutes, washed with Ca^{2+}-free Tyrode solution, and stored at 4 °C in PBS containing 0.02% $NaCN_3$. For immunolabeling, the cells were spun (250 rpm, 5 min) onto silanized slides (Dako) using a cytospin (Life Science International) and were blocked in 0.5% BSA (15 min) and incubated with the anti-SERCA2a (BioReagents) for 2 h. The samples were then treated with CY3-conjugated secondary antibody for 1 h. Stained cells were examined by confocal laser scanning microscopy using a Leica TCS SP equipped with an argon/krypton and UV laser with the appropriate filter spectra adjusted for the detection of CY3 fluorescence.

Western blot of the expression of sarcoplasmic reticulum Ca^{2+}-ATPase

Control and TNFα-treated cells from 6 rabbits were centrifuged and washed with cold PBS and lysed on ice for 30 minutes in an RIPA buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktails (Sigma-Aldrich Corp.). The protein concentration was determined with a Bio-Rad protein assay reagent (Bio-Rad). For the immunoblot of the SERCA2a, protein (2.5 μg) was resolved on 8% SDS-polyacrylamide gels, then transferred to Hybond-P polyvinylidene difluoride membranes (Amersham). After blocking with 5% nonfat milk for one hour, we incubated the membranes with mouse monoclonal antibody against SERCA2a (1:5000 dilution; Affinity Bioreagents, Golden, CO) overnight at 4 °C, washed 3 times with phosphate-buffered saline with 0.5% Tween 20 and incubated with secondary antibody for one hour. The membranes were analyzed with chemiluminescence reagent (Millipore). The band intensities were quantified with densitometric analysis by the use of the Image-Pro Plus software. The targeted bands were normalized with cardiac α-sarcomeric actin (Sigma-Aldrich Corp.).

Statistical analysis

The continuous variables are expressed as mean±S.E.M. The differences between the PV cardiomyocytes with or without administrating TNFα were analyzed by unpaired T test. Nominal variables were compared by a Chi-square analysis with Yates correction or Fisher's exact test. A P value lower than 0.05 was considered to be statistically significant.

Results

Effect of TNFα on the electrical activity of the PV cardiomyocytes

Fig. 1 shows the tracings of the action potentials in the control and TNFα-treated PV cardiomyocytes. The spontaneous beating rates (2.1±0.1 Hz versus 2.3±0.2 Hz) were similar between the control (n=45) and TNFα-treated (n=28) PV cardiomyocytes. The TNFα-treated PV cardiomyocytes tended to have a higher incidence of delayed afterdepolarizations (10 of 18, 56%) compared with that of the control PV cardiomyocytes (10 of 32, 31%, P=0.09). In addition, the TNFα-treated PV cardiomyocytes had larger amplitudes of the delayed afterdepolarization than the control PV cardiomyocytes (6.0±1.7 mV versus 2.6±0.8 mV, P<0.05). The delayed afterdepolarization may induce a triggered beat (Fig. 1B). Moreover, the APD_{20} (30±2 ms versus 37±2 ms, P<0.05) and APD_{50} (52±3 ms versus 64±3 ms, P<0.05) in the TNFα-treated (n=12) were shorter than those in the control (n=21) PV cardiomyocytes, but the APD_{90} (92±4 ms

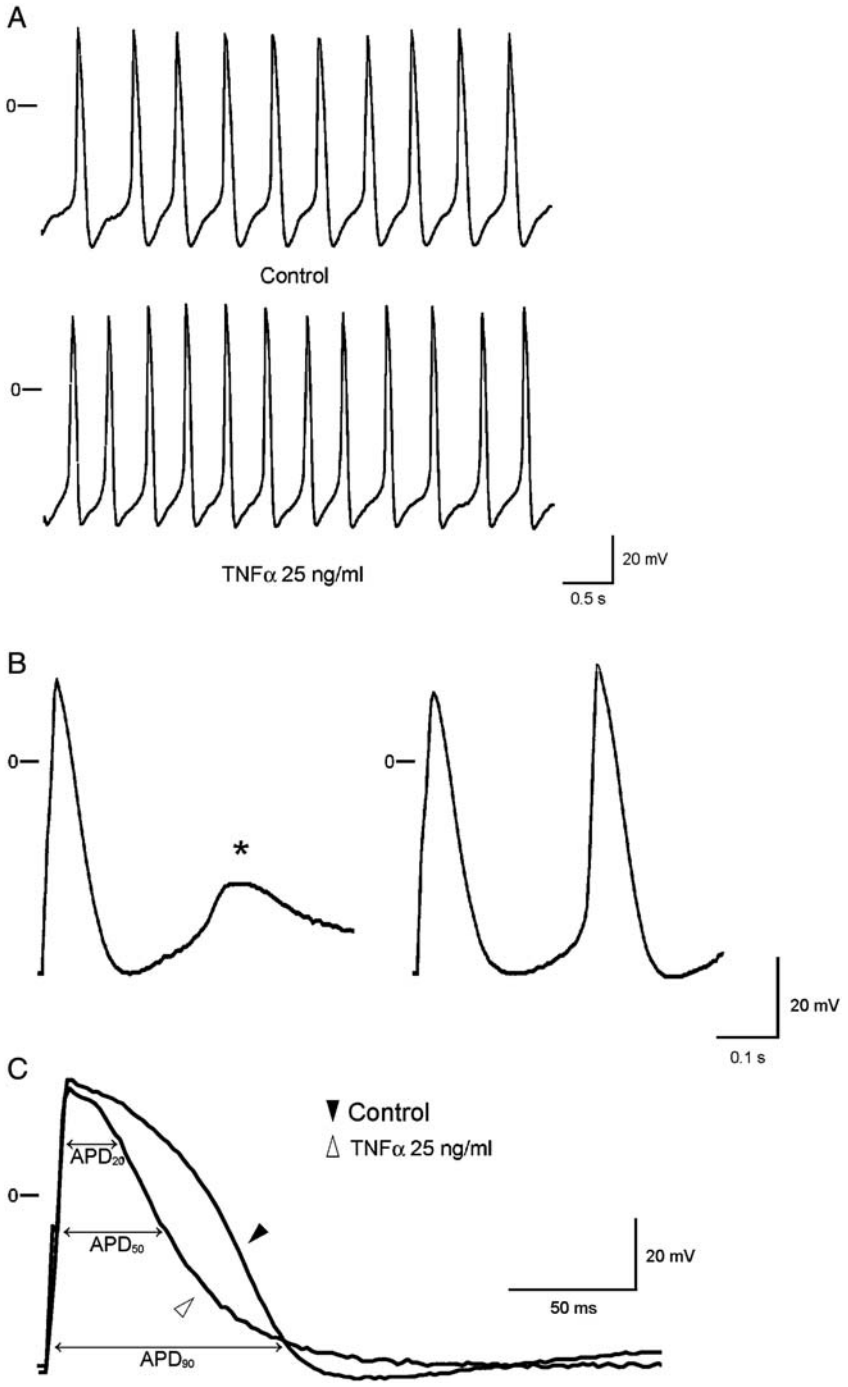

Fig. 1. Action potentials of the control and TNFα-treated PV cardiomyocytes. Panel A. Beating rates were similar between the control and TNFα-treated PV cardiomyocytes. Panel B shows the tracings of the occurrence of a delayed afterdepolarization (*) with a triggered beat during electrical stimulation in a TNFα-treated PV cardiomyocyte. Panel C shows the superimposed tracings of the action potential (elicited by 2 Hz electrical stimulation) of the control (▼) and TNF-α-treated (△) PV cardiomyocytes. The APD_{20} and APD_{50} in the TNFα-treated PV cardiomyocytes were shorter than those in the control PV cardiomyocytes.

versus 104±3 ms,$P>0.05$) and resting membrane potential (−58±1 mV versus −61±2 mV) were not different between the two groups (Fig. 1C).

Effect of TNFα on the membrane currents of the PV cardiomyocytes

Fig. 2 shows the tracings and *I–V* relationship of the $I_{Ca\text{-}L}$ in the two groups. The current density of $I_{Ca\text{-}L}$ in the TNFα-treated cardiomyocytes was significantly smaller than that of the control PV cardiomyocytes. In contrast, as the examples show in Fig. 3A, the current density of the I_{to} in the TNFα-treated was larger than that in the control PV cardiomyocytes. Moreover, the control and TNFα-treated PV cardiomyocytes had a similar current density of the I_K (Fig. 3B). Fig. 4 shows the recordings and *I–V* relationship of the I_{K1} in the TNFα-treated and control PV cardiomyocytes. The current density of the I_{K1} was similar between the two groups.

As the examples show in Fig. 5, the TNFα-treated ($n=24$) PV cardiomyocytes had larger transient inward currents than

the control ($n=44$) PV cardiomyocytes (1.11±0.18 pA/pF vs. 0.69±0.09 pA/pF, $P<0.05$). Fig. 6 shows the tracings and I–V relationship of the nickel-sensitive NCX currents in both groups. The TNFα-treated PV cardiomyocytes had larger nickel-sensitive NCX currents than the control PV cardiomyocytes.

Effect of TNFα on the intracellular calcium of the PV cardiomyocytes

The TNFα-treated PV cardiomyocytes had smaller amplitudes of the ratio of the $[Ca^{2+}]_i$ transient than the control PV cardiomyocytes at a stimulation frequency of 1.5 Hz (Fig. 7). The TNFα-treated and control PV cardiomyocytes had similar ratios of the peak of the $[Ca^{2+}]_i$ transient. However, the TNFα-treated PV cardiomyocytes had a larger ratio of the diastolic $[Ca^{2+}]_i$ than the control PV cardiomyocytes. The TNFα-treated PV cardiomyocytes had a longer decay portion of the $[Ca^{2+}]_i$ transient than the control PV cardiomyocytes (Fig. 7D). Moreover, the sarcoplasmic reticulum Ca^{2+} content in the TNFα-treated PV cardiomyocytes was smaller than that in the control PV cardiomyocytes (Fig. 7E and F).

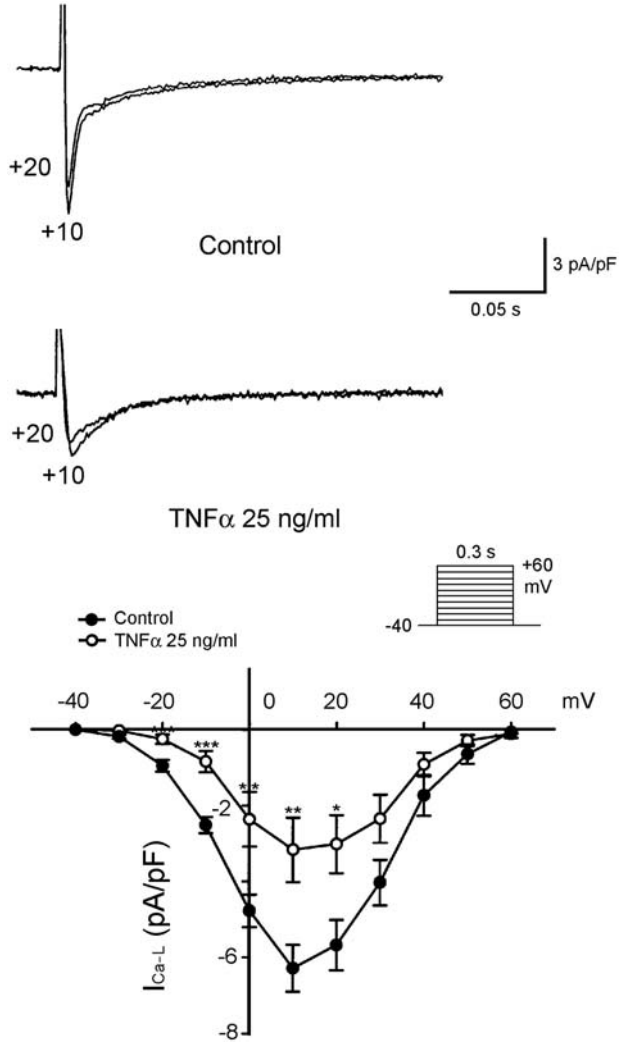

Fig. 2. The current tracings and I–V relationship of the I_{Ca-L} of the control and TNFα-treated PV cardiomyocytes. The I_{Ca-L} in the TNFα-treated PV cardiomyocytes ($n=8$) was smaller than that in the control ($n=10$) PV cardiomyocytes. The insets of the current tracings show the various clamp protocols. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ versus the control PV cardiomyocytes.

Effect of TNFα on the SERCA2a expression of the PV cardiomyocytes

An immunocytochemical investigation was performed to examine the expression of SERCA2a in the control and TNFα-treated PV cardiomyocytes. In the control PV cardiomyocytes, an intense immunostaining of the SERCA2a was detected at the cell periphery in close apposition to the surface membrane. In the TNFα-treated PV cardiomyocytes, there was a similar distribution of the SERCA2a, however, the staining was less intense (Fig. 8). Western blot experiments showed a significant reduction in the SERCA2a protein expression in the TNFα-treated PV cardiomyocytes (Fig. 8B).

Discussion

Effects of TNFα on the electrical activity of the PV cardiomyocytes

In this study, we found that TNFα enhanced the triggered activity of the PV cardiomyocytes through increasing the delayed afterdepolarizations. Based on these findings, we suggest that TNFα plays an arrhythmogenic role in the PVs. TNFα's proarrhythmogenic effects on the PV cardiomyocytes may contribute to the higher incidence of atrial fibrillation when patients suffer from inflammation or undergo cardiac surgery (Zaman et al., 2000; Chung et al., 2001; Seguin et al., 2004).

Congestive heart failure has been found to change the atrial electrophysiology and result in a higher incidence of atrial fibrillation (Li et al., 2000). In patients with heart failure, TNFα in the circulation is elevated and their serum concentration is correlated with the disease severity (Levine et al., 1990; Latini et al., 1994). Therefore, the proarrhythmic effects of the TNFα may increase the PV arrhythmogenic activity, thereby causing atrial fibrillation during heart failure. Since TNFα can shorten the APD_{20} and APD_{50}, we suggest that TNFα can facilitate the genesis of a microreentrant circuit and induce PV-related arrhythmia because reentrant circuits in PVs have been found to cause the PV arrhythmogenicity (Chen et al., 2000; Hocini et al., 2002).

The heart has been found to synthesize TNFα under stress (Kapadia et al., 1995). In the failing human hearts, intracardiac TNFα level may be significantly higher than serum TNFα level (Torre-Amione et al., 1996). Moreover, serum TNFα level is drastically elevated (ranged from 0.1 ng/ml to 5 ng/ml) during sepsis (Damas et al., 1989). Based on these findings, we believe that the dosage of TNFα used in this experiment is clinically relevant.

Effects of TNFα on the membrane currents of the PV cardiomyocytes

In this experiment, we found that TNFα decreased the I_{Ca-L} significantly in the PV cardiomyocytes as compared with the

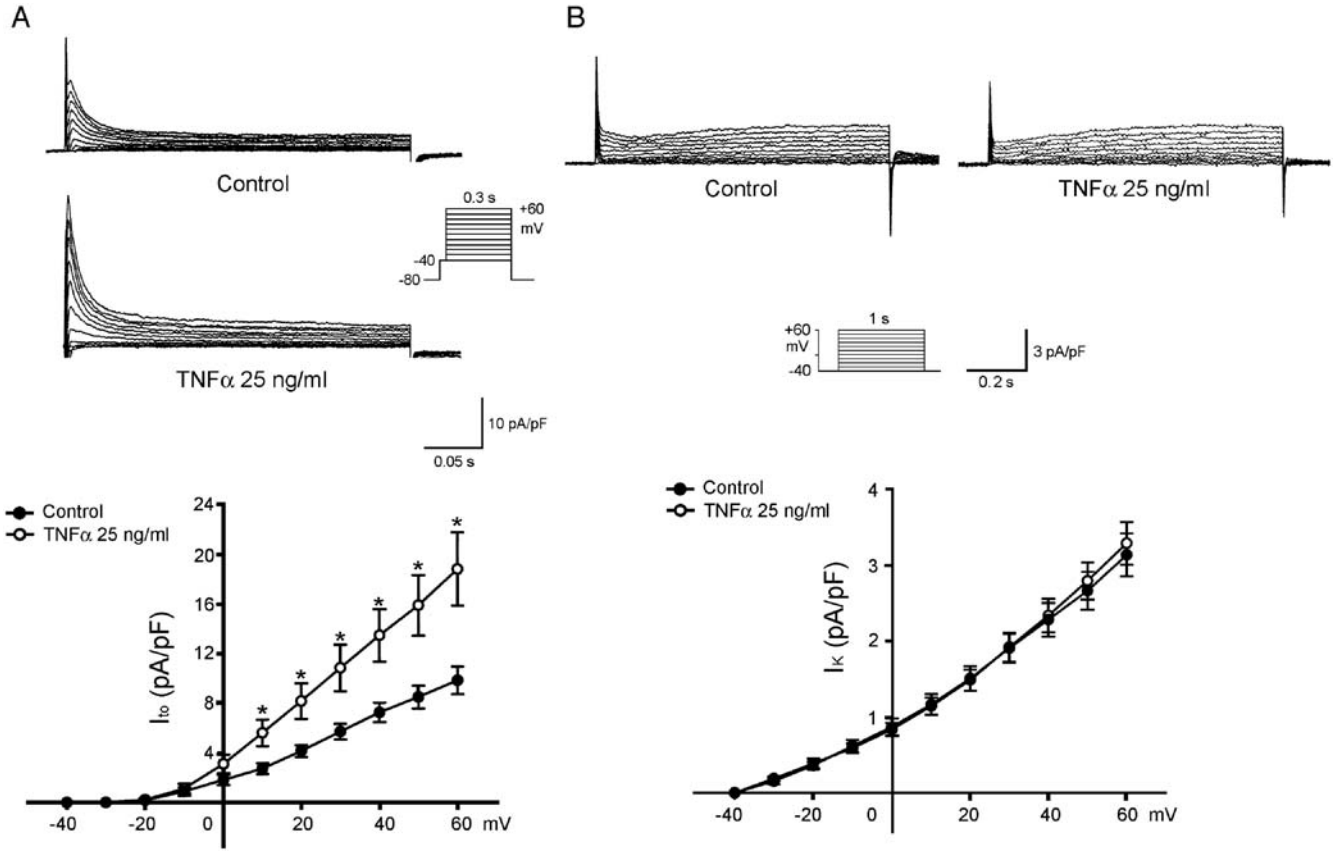

Fig. 3. Effects of TNFα on the I_{to} and I_K in the PV cardiomyocytes. Panel A shows the current tracings and I–V relationship of the I_{to} of the control and TNFα-treated PV cardiomyocytes. The I_{to} in the TNFα-treated PV cardiomyocytes ($n=10$) was larger than that in the control PV cardiomyocytes ($n=10$). Panel B shows the current tracings and I–V relationship of the I_K in the control and TNFα-treated PV cardiomyocytes. The I_K was similar between the TNFα-treated ($n=17$) and control ($n=14$) PV cardiomyocytes. The insets of the current traces show the various clamp protocols. *$P<0.05$, versus the control PV cardiomyocytes.

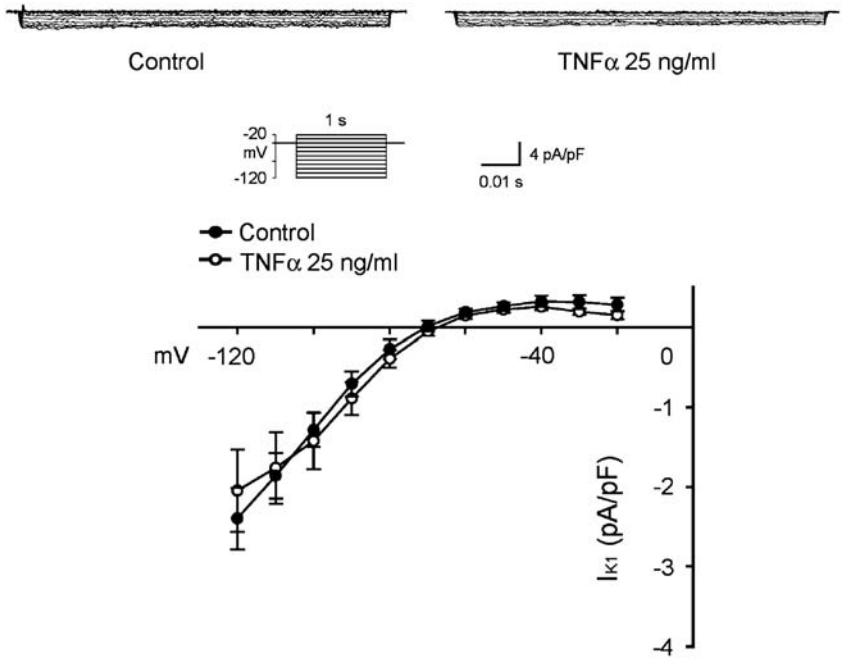

Fig. 4. The current tracings and I–V relationship of I_{K1} of the control and TNFα-treated PV cardiomyocytes. The I_{K1} was similar between the TNFα-treated ($n=9$) and control ($n=11$) PV cardiomyocytes. The insets of the current tracings show the various clamp protocols.

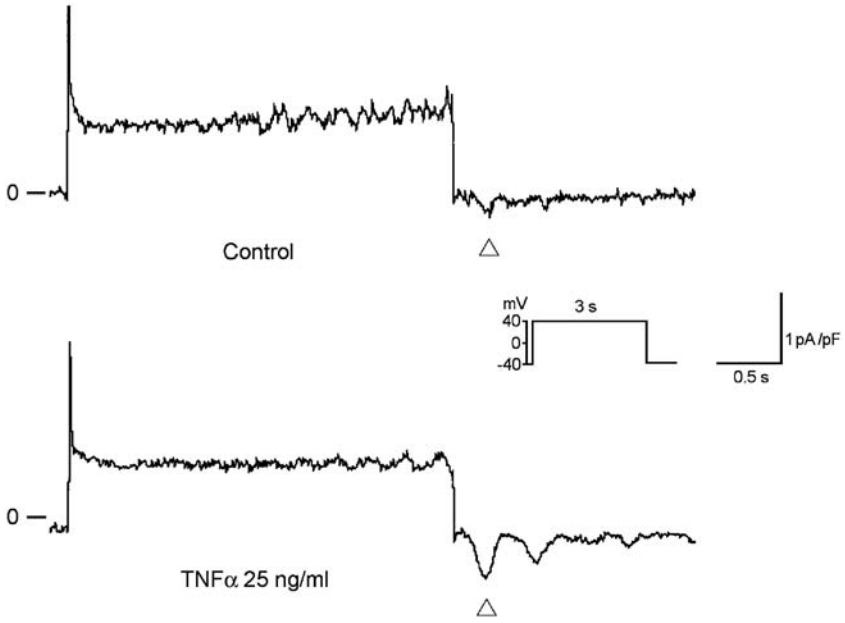

Fig. 5. Effects of TNFα on the transient inward current of the PV cardiomyocytes. The transient inward current (△) in the TNFα-treated PV cardiomyocyte was larger than that in the control PV cardiomyocyte. The insets of the current tracings show the clamp protocols.

control cells. This finding is similar to the known effects of TNFα on ventricular myocytes (Krown et al., 1995). The decrease in the $I_{Ca\text{-}L}$ caused by TNFα has been suggested to reduce myocytes' contractility during sepsis. This effect may also cause a decrease in the $[Ca^{2+}]_i$ transient. Additionally, the increase in the I_{to} and the decrease in the $I_{Ca\text{-}L}$ by TNFα can shorten APD_{20} and APD_{50}. However, the current densities of the I_K and I_{K1} were similar in both the control and TNFα-treated PV cardiomyocytes, resulting in a similar resting membrane potential and APD_{90} in the two groups.

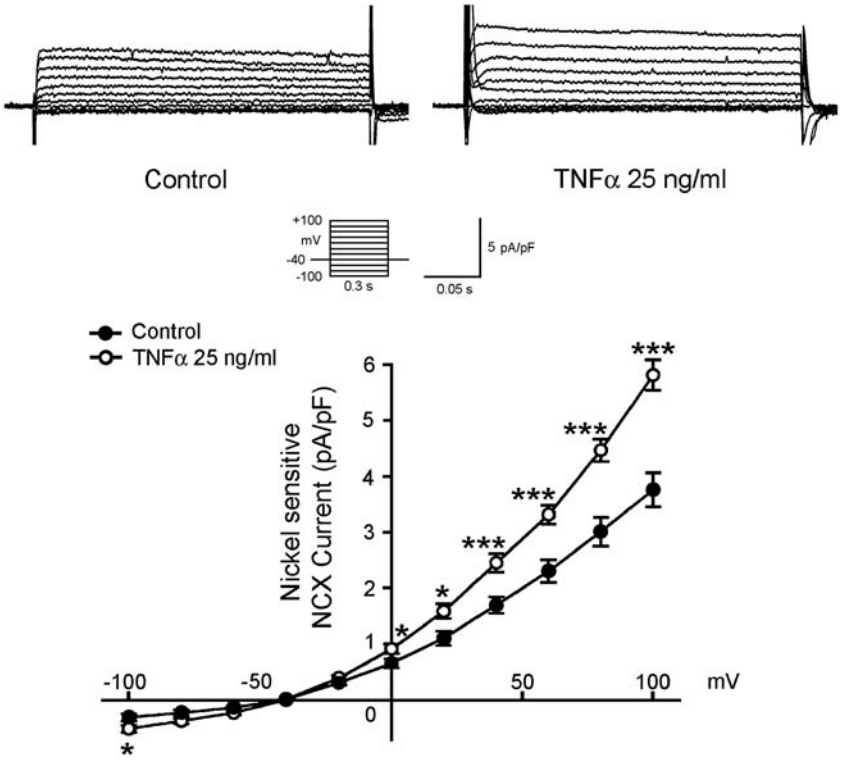

Fig. 6. The current tracings and *I–V* relationship of the nickel sensitive NCX currents of the control and TNFα-treated PV cardiomyocytes. The nickel sensitive NCX currents in the TNFα-treated PV cardiomyocytes ($n=11$) were larger than those in the control PV cardiomyocytes ($n=12$). The insets of the current tracings show the various clamp protocols. *$P<0.05$, ***$P<0.005$ versus the control PV cardiomyocytes.

The transient inward current can induce triggered activity and cause the PV arrhythmogenicity (Chen et al., 2001, 2002a,b). The results of this study showed that the transient inward current and NCX currents of the PV cardiomyocytes were significantly increased by TNFα as compared with the control cells. NCX currents can cause transient inward current and delayed afterdepolarizations in cardiomyocytes. Based on those results, we suggest that TNFα may alter the NCX currents and change the transient inward current with the enhanced PV arrhythmogenicity. In addition, the effect of TNFα may also underlie the increase of NCX currents during heart failure (Hobai and O'Rourke, 2000).

Effect of TNFα on the intracellular calcium of the PV cardiomyocytes

TNFα has been shown to decrease the $[Ca^{2+}]_i$ transient and systolic $[Ca^{2+}]_i$ (Yokoyama et al., 1993; Krown et al., 1995; Goldhaber et al., 1996; Cailleret et al., 2004). Whether TNFα regulates $[Ca^{2+}]_i$ in cardiac pacemaker cells to induce arrhythmias is not clear. In this study, we found that TNFα significantly decreased the $[Ca^{2+}]_i$ transient, but increased the diastolic $[Ca^{2+}]_i$ in the PV cardiomyocytes. These findings may cause TNFα's negative inotropic effects to impair heart function during sepsis or heart failure. Moreover, the results of this study showed that τ_{Ca} was longer in the TNFα-treated PV cardiomyocytes, indicating that TNFα can slow the $[Ca^{2+}]_i$ reuptake, which is the function of NCX currents and SERCA2a. Therefore, the impaired decline of $[Ca^{2+}]_i$ is mainly caused by the abnormal SERCA2a function because there were increased NCX currents in the TNFα-treated PV cardiomyocytes. To the best of our knowledge, this study is the first time to demonstrate that TNFα has a direct proarrhythmic potential through its effect on $[Ca^{2+}]_i$ handling. The result of this study is similar to the known effects of heart failure on the $[Ca^{2+}]_i$ transient, where the $[Ca^{2+}]_i$ transient is reduced, with impaired

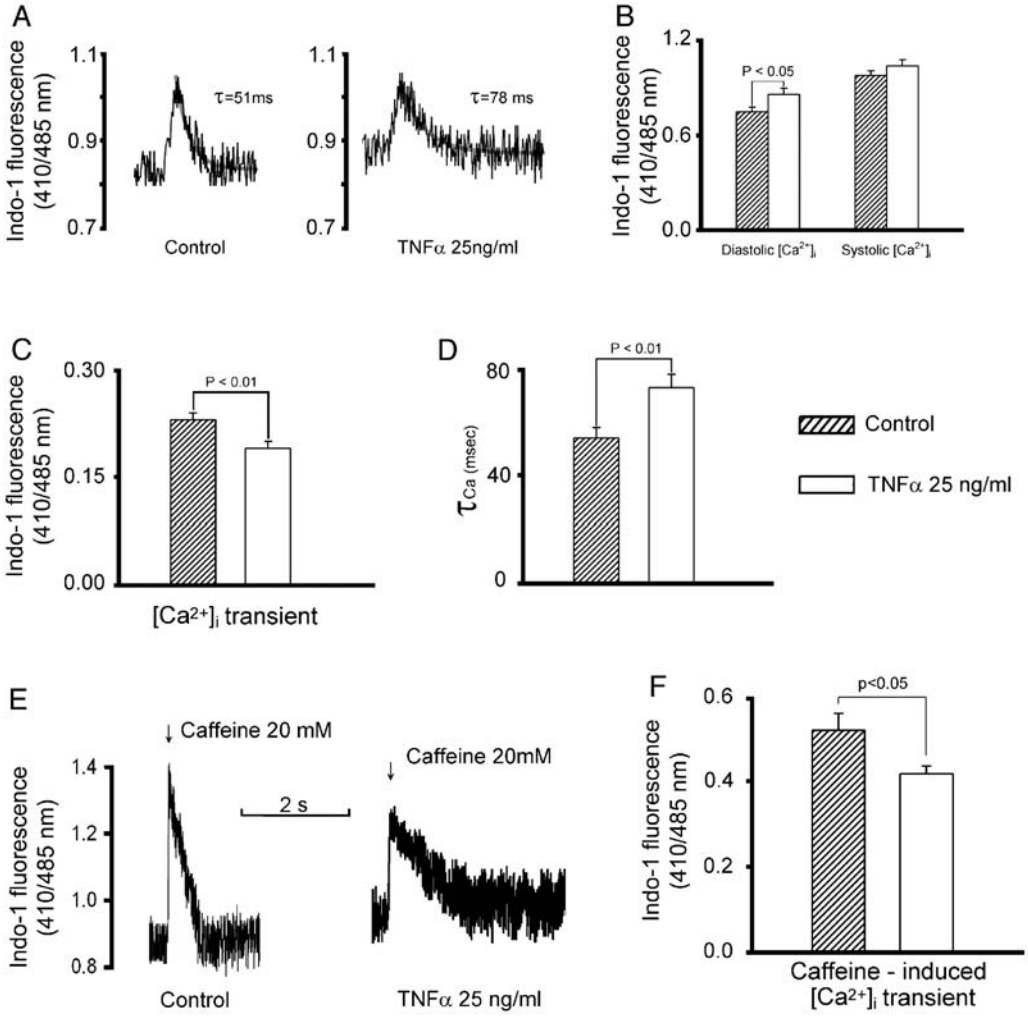

Fig. 7. Effects of TNFα on the $[Ca^{2+}]_i$ and sarcoplasmic reticulum Ca^{2+} content of the PV cardiomyocytes. Panel A shows the tracings of the $[Ca^{2+}]_i$ transient in a field-stimulation of 1.5 Hz in the control and TNFα-treated PV cardiomyocytes. Panels B–D show the average of the ratios of the diastolic $[Ca^{2+}]_i$, peak systolic $[Ca^{2+}]_i$ (panel B), $[Ca^{2+}]_i$ transient (panel C), and decay portion of the ratio of the $[Ca^{2+}]_i$ transient (panel D) from the TNFα-treated ($n=18$) and control ($n=23$) PV cardiomyocytes, respectively. Panel E shows the tracings of caffeine-induced $[Ca^{2+}]_i$ transient. Panel F shows the average of sarcoplasmic reticulum Ca^{2+} content from the TNFα-treated ($n=10$) and control ($n=10$) PV cardiomyocytes.

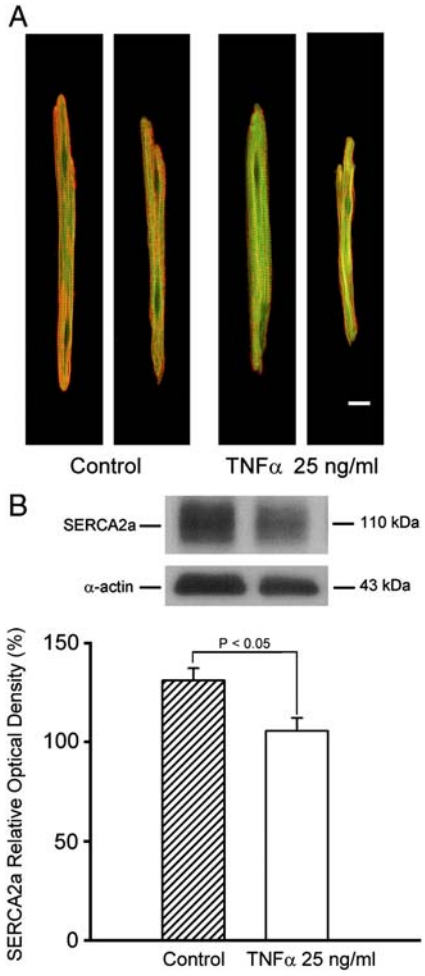

Fig. 8. Confocal micrographs and western blot of the expression of SERCA2a in the control and TNFα-treated PV cardiomyocytes. Panel A. Red spots show the immunostaining of SERCA2a. The cells were counterstained with phalloidin. Bar=20 μm. Panel B. Western blot shows the alternation of SERCA2a protein levels from the control and TNFα-treated groups (n=6 for each condition).

SERCA2a and increased NCX currents (Bers et al., 2003). Taken together, we suggest that TNFα may mediate the electrophysiological changes of cardiomyocytes in heart failure, and that the impaired calcium reuptake due to TNFα-induced reduction in the SERCA2a may increase the diastolic $[Ca^{2+}]_i$ thereby causing arrhythmias.

Through immunolabeling, we showed that TNFα significantly decreased the expression of SERCA2a in the PV cardiomyocytes as compared with the control cells. The decrease in the SERCA2a caused by TNFα can impair calcium reuptake and lengthen the decay of the $[Ca^{2+}]_i$ transient with increased diastolic $[Ca^{2+}]_i$. Delayed afterdepolarizations might arise from spontaneous Ca^{2+} release due to a Ca^{2+} overloaded sarcoplasmic reticulum. However, during the decrease of SERCA2a, the NCX activity needs to be increased because NCX and SERCA2a compete with each other for the Ca^{2+} removal within the cells. Therefore, the enhancing NCX could cause delayed afterdepolarizations in spite of the decrease in the $[Ca^{2+}]_i$ transients and sarcoplasmic reticulum Ca^{2+} content. These findings suggest that TNFα may induce cardiac arrhythmias through its effects on the SERCA2a. PV cardiomyocytes have the electrophysiological characteristics of decreased I_{K1} and less negative resting membrane potentials (Chen et al., 2001, 2002a, b; Melnyk et al., 2005). Thus, abnormal calcium regulation can easily trigger PV arrhythmogenesis due to a reduced depolarizing threshold. These results also suggest that this experimental model may be useful in studying the mechanisms of heart failure-induced cardiac arrhythmias.

The data should be interpreted with caution due to the limitations of this study. First, the TNFα arrhythmogenic potentials may have been underestimated because these cells were only incubated with TNFα for 7 to 10 h to maintain the viability of the PV cardiomyocytes. Second, the threshold of TNFα effectiveness was still undetermined through the single concentration TNFα experiment in this study. And third, we did not have the absolute values of $[Ca^{2+}]_i$ in this experiment and we only used fluorescence ratio as an index of $[Ca^{2+}]_i$. An absolute calibration of the fluorescent Ca^{2+} indicators for the $[Ca^{2+}]_i$ may have involved a lot of uncertainties (Bassani et al., 1995).

Conclusions

This study demonstrated that TNFα increases the PV arrhythmogenic activity and impairs the calcium regulation in the PV cardiomyocytes. These results may account for the higher incidence of atrial fibrillation in patients during sepsis, heart failure, and clinical status of post cardiac surgery.

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