TNF- Rapidly Antagonizes the β -Adrenergic Responses of the Chloride Current in Guinea-Pig Ventricular Myocytes

Kenji Iino, MD; Hiroyuki Watanabe, MD; Takashi Saito, MD; Satoshi Kibira, MD; Toshihiko Iijima, MD*; Mamoru Miura, MD

The purpose of this study was to test the hypothesis that tumor necrosis factor- α (TNF- α) rapidly antagonizes the β -adrenergic responses of the chloride current and to clarify the intracellular mechanisms responsible for the anti-adrenergic action. The whole-cell patch-clamp technique was used to monitor the anti-adrenergic effects of TNF- α on the cAMP-dependent chloride current (I_{Cl}) recorded from isolated guinea-pig ventricular myocytes. Ramp pulses (± 120 mV; $dv/dt = \pm 0.4$ V/s) were applied from the holding potential of -40 mV. TNF- α rapidly (< 15 min) inhibited the isoproterenol (Iso, $0.1 \mu\text{mol/L}$)-induced I_{Cl} in a concentration-dependent manner (30 – $1,000$ U/ml, $IC_{50} = 144$ U/ml, $n = 30$). The inhibitory action of TNF- α was also observed when I_{Cl} had been previously stimulated by $1 \mu\text{mol/L}$ forskolin ($n = 5$). Prior exposure of myocytes to $5 \mu\text{g/ml}$ pertussis toxin (PTX) hardly affected the anti-adrenergic action of TNF- α ($n = 4$). However, when I_{Cl} was induced by both 8-bromo-cAMP ($100 \mu\text{mol/L}$) and isobutylmethylxanthine (0.1 mmol/L), TNF- α ($1,000$ U/ml) failed to decrease I_{Cl} amplitude ($n = 5$). Prior exposure of myocytes to 5 mg/ml pertussis toxin (PTX) hardly affected the anti-adrenergic action of TNF- α ($n = 4$). Furthermore, despite the presence of nitro-L-arginine methyl ester (0.1 mmol/L), a nitric oxide synthase (NOS) inhibitor, TNF- α reversed the Iso-induced increase in I_{Cl} ($n = 5$). These results suggest that TNF- α rapidly antagonizes the β -adrenergic responses of I_{Cl} by reducing cAMP concentration. This anti-adrenergic action is mediated by neither the PTX-sensitive G proteins regulatory pathway nor constitutive NOS activation. (Circ J 2003; 67: 347–353)

Key Words: Anti-adrenergic action; Chloride channel; Cyclic AMP; Guinea-pig heart; TNF-

Tumor necrosis factor- α (TNF- α) is a proinflammatory cytokine with a broad range of pleiotropic effects.¹ The adult human heart expresses both mRNA and receptor proteins for type I and type II TNF-receptors.² TNF- α is also expressed de novo by cardiac myocytes after certain forms of stress³ and plays a fundamental role in various pathophysiological conditions, including acute myocarditis, myocardial infarction, sepsis-associated cardiac dysfunction, and advanced congestive heart failure.^{4–7}

It seems likely that different processes are responsible for the early and late cardiac effects of TNF- α .^{8,9} Excessive production of inducible nitric oxide synthase (iNOS) has been shown to cause the late onset of functional depression in the heart^{9–11} but the mechanisms underlying the early onset of cardiac depression remain controversial.

Finkel et al postulated that TNF- α depressed contractility of isolated papillary muscles within 5 min and that the effects were reversed by NG-monomethyl-L-arginine, speculating that the early cardiodepressant effects of TNF- α were mediated by a constitutive NOS (cNOS) action in the myocardium.¹² Krown et al have reported that TNF- α rapidly inhibits the basal L-type Ca^{2+} current and contrac-

tile Ca^{2+} transients in rat cardiac myocytes.¹³ A more recent report has also demonstrated that sphingosine mediates the immediate negative inotropic effects of TNF- α in adult feline cardiac myocytes.¹⁴ As mentioned earlier, although a large number of studies have been done on the intracellular signaling mechanisms that contribute to the early cardiac effects of TNF- α , the modulation of the β -adrenergic responses, including the ion channel regulation, remains unclear. The purpose of this study was to test the hypothesis that TNF- α rapidly antagonizes the β -adrenergic responses, and to provide additional information about the intracellular mechanisms underlying the rapid anti-adrenergic action of TNF- α .

In cardiac myocytes, activation of β -adrenergic receptors stimulates the activity of a number of different ion currents, including those conducted by L-type Ca^{2+} channels and cAMP-dependent Cl^- channels. Although there is a report showing TNF- α -induced direct inhibition of L-type Ca^{2+} channels,¹³ the modulation of cAMP-dependent Cl^- channels has never been studied. The activation mechanism of the Cl^- channels is well known: the response is mediated by a cAMP-dependent cascade, which leads from the agonist–receptor binding to the final phosphorylation of the channel protein.^{15–18} Therefore we monitored the cAMP-dependent Cl^- channels as a reliable parameter of β -adrenergic regulation.

(Received March 27, 2002; revised manuscript received December 20, 2002; accepted January 6, 2003)

The 2nd Department of Internal Medicine and *Department of Pharmacology, Akita University School of Medicine, Akita, Japan
Mailing address: Takashi Saito, MD, The 2nd Department of Internal Medicine, Akita University School of Medicine, Hondo 1-1-1, Akita 010-8543, Japan. E-mail: tsaito@im2.med.akita-u.ac.jp

Methods

Cell Preparation

Single ventricular cells of adult guinea-pig hearts were obtained by an enzymatic dissociation method.¹⁹ The preparation of single cells and the technique of whole-cell recording are essentially the same as previously reported.²⁰ Briefly, the heart was perfused through the coronary arteries with Ca^{2+} -free Tyrode's solution containing 0.04% (72.8 units/ml) collagenase (Wako Pure Chemical Industry, Osaka, Japan) for 25–30 min at 36°C. The heart was then stored in the storage solution²¹ at 4°C for at least 1 h. A small piece of the ventricular tissue was dissected and agitated gently in a recording chamber (0.7 ml in volume) filled with normal Tyrode's solution. The recording chamber was perfused at 2 ml/min with normal Tyrode's solution after the cell had settled on the floor of the chamber. Experiments were carried out at 36–37°C on rod-shaped quiescent single cells that had the clear striation of sarcomeres. The investigation conforms 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 1985).

Solutions and Drugs

The composition of normal Tyrode's solution was (in mmol/L): 136.9 NaCl; 5.4 KCl; 1.8 CaCl_2 ; 0.53 MgCl_2 ; 0.33 NaH_2PO_4 ; 5.0 HEPES; 5.5 glucose; and the pH was adjusted to 7.4 with NaOH. The standard external solution for recording the Cl^- current contained (in mmol/L): 140 NaCl; 2 BaCl_2 ; 2 MgCl_2 ; 5.0 HEPES; 0.02 ouabain; 0.001 nifedipine, and the pH was adjusted to 7.4 with CsOH ($[\text{Cl}]_o = 148 \text{ mmol/L}$). The low Cl^- external solution was made by replacing 99 mmol/L NaCl with an equimolar Na-aspartate ($[\text{Cl}]_o = 45 \text{ mmol/L}$). The storage solution contained (in mmol/L): 10 taurine; 10 oxalic acid; 70 glutamic acid; 25 KCl; 10 KH_2PO_4 ; 0.5 EGTA; 11 glucose; and 10 HEPES (pH=7.4, adjusted with KOH). The internal solution contained (in mmol/L): 90 Cs-aspartate; 15 CsCl; 5 MgCl_2 ; 20 tetraethyl ammonium chloride (TEA); 5 creatine phosphate disodium salt; 5 MgATP; 5.0 EGTA; and 5 HEPES (pH=7.2, adjusted with CsOH, $[\text{Cl}]_i = 45 \text{ mmol/L}$). Na2GTP (0.1 mmol/L) was added to the internal solution to reduce the possible influence of current run-down.

Human recombinant TNF- (Research Biochemicals International, Natick, MA, USA) was dissolved in distilled water at a concentration of $19.2 \times 10^4 \text{ U/ml}$ as a stock solution, and was diluted to various concentrations in the external solutions just before the experiments. Isoproterenol (Iso, Wako Pure Chemical Industry) was prepared as an aqueous stock solution containing an equimolar concentration of ascorbic acid. Nifedipine (Wako Pure Chemical Industry) was prepared as a stock solution in ethanol at a final concentration of 0.03%. Forskolin (Wako Pure Chemical Industry) was prepared as a stock solution in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1%. Nitro-L-arginine methyl ester (L-NAME) and pertussis toxin (PTX) were obtained from Sigma Chemical Co (St Louis, MO, USA). Isobutyl-methylxanthine (IBMX) was purchased from Wako Pure Chemical Industry, and carbachol and 8-bromo-cAMP were from Calbiochem Co (La Jolla, CA, USA).

The recording chamber was coated with silicon to prevent TNF- from sticking to the chamber surfaces.

Fig1. Chloride current stability. (A) Activation of I_{Cl} by isoproterenol (Iso, 0.1 $\mu\text{mol/L}$). The superimposed current traces recorded at control, Iso (1 min), and Iso (15 min) are shown. The horizontal bar indicates the zero current level. (B) The representative I–V curve of Iso-induced I_{Cl} is shown. Iso-induced I_{Cl} was obtained by subtracting the current trace recorded under control conditions from that recorded in the presence of 0.1 $\mu\text{mol/L}$ Iso. (C) Iso-induced I_{Cl} was obtained by subtracting the current trace recorded under control conditions from that recorded in the presence of 0.1 $\mu\text{mol/L}$ Iso. Iso-induced I_{Cl} at +78 mV was normalized to the level recorded during initial exposure to Iso, and plotted against time. The horizontal bar above the graph indicates extracellular application of Iso (0.1 $\mu\text{mol/L}$).

Voltage Clamp and Recording Technique

The single-pipette whole-cell recording technique was used.²² The electrode resistance ranged from 2 to 4 M Ω when filled with the internal solution. After the formation of the gigaohm seal, the patch of membrane was disrupted by suction of -50 to $-100 \text{ mmH}_2\text{O}$. Current and voltage signals were directly stored on the hard disc of a computer running pClamp software (version 6.0.3, Axon, USA). The Cl^- current was isolated by inhibiting all K^+ channel currents with Cs^+ -containing and/or TEA-containing internal and external solutions. The L-type Ca^{2+} current was blocked by adding 1 $\mu\text{mol/L}$ nifedipine to all external solutions. For recording I_{Cl} , a ramp pulse ($\pm 120 \text{ mV}$; $\text{dV/dt} = \pm 0.4 \text{ V/s}$) was employed with the holding potential of -40 mV . The negative limb of the ramp pulse was used to plot the I–V curves. I_{Cl} was determined by subtraction of the current prior to the agonist application from the agonist-induced current. The liquid junctional potential between the internal and external solutions was -6 mV . Therefore, all values of potentials given in these experiments were corrected.

Fig2. Effect of TNF- on Iso-induced I_{Cl} . (A) The superimposed current traces recorded at control, 0.1 μmol/L Iso (1 min), and 0.1 μmol/L Iso plus 1,000 U/ml TNF- are shown. The horizontal bar indicates the zero current level. (B) For each trial, I_{Cl} at +78 mV was normalized to the maximum level recorded during that trial. The relative currents were plotted against time. The horizontal bars above the graph indicate the exposure to Iso (0.1 μmol/L) and TNF- (1,000 U/ml). (C) The I-V curve of TNF- sensitive I_{Cl} is obtained by subtracting the current in the presence of Iso plus TNF- from the Iso-induced current. The negative limb of the ramp pulse was used to plot the I-V curve.

Data Analysis

Values are expressed as means ± standard error of the mean of *n* observations, where *n* represents the number of cell experiments. Statistical significance was evaluated by Student's unpaired *t* test where appropriate. Differences with values of *p* < 0.05 were considered to be significant. IC_{50} was determined after fitting the concentration-response curve according to a Hill's equation:

$$I = 1 - 1 / \{1 + (IC_{50}/X)^n\}$$

where *I* is the relative I_{Cl} ; *X* is the concentration of TNF-; and *n* is the Hill's coefficient.

Results

Chloride Current Stability

It is known that the effect of Iso on I_{Cl} declines with time (so-called run down)²³ and experimental conditions, such as pipette resistance or intracellular GTP concentration, are implicated in the run down.²⁴ We first evaluated the time-dependent decline of Iso-induced I_{Cl} . When the cell was exposed to the external solution containing 0.1 μmol/L Iso, enhancement of membrane currents accompanied by an

Fig3. The concentration-response relationship for the effects of TNF- on Iso-induced I_{Cl} . (A) The superimposed current traces recorded at control, 0.1 μmol/L Iso plus 300 U/ml TNF- are shown. The horizontal bar indicates the zero current level. (B) Chart recording of the membrane current is shown. Bars at the top indicate extracellular application of Iso (0.1 μmol/L) and TNF- (300 U/ml). Note that the inhibitory effect of TNF- reached the steady-state at 6 min. (C) The coordinate axis of the histogram shows the Iso-induced relative currents at +78 mV after exposure to various TNF- concentrations. Each bar represents the result obtained from 5–10 cells. Bar heights are means and brackets indicate ± standard error of the mean. IC_{50} was determined after fitting the concentration-response curve according to a Hill's equation (IC_{50} = 144 U/ml).

inward shift of the holding current was observed (Fig 1A).

The I-V curve of the Iso-activated membrane currents displayed weak outward rectification and reversed at -27 mV (Fig 1B), which is close to the equilibrium potential for Cl^- ($E_{Cl} = -30$ mV), indicating that the Iso-induced currents were carried by Cl^- . The Iso-induced I_{Cl} measured at +78 mV was normalized to the level recorded during initial exposure to Iso and the time course of the change is shown in Fig 1C. The response to 0.1 μmol/L Iso appeared immediately (~40 s) and was well maintained for more than 15 min (percent run down: 10.7 %, *n* = 10). A more quantitative analysis found the magnitude of the Iso-induced I_{Cl} preserved up to 89.3% of the initial response after 17 ± 2 min (*n* = 10).

Effects of TNF- on Iso-Induced I_{Cl}

To determine whether TNF- can antagonize the adrenergic responses of I_{Cl} , TNF- (1,000 U/ml) was applied to the external solution after the effect of Iso had been observed. TNF- (1,000 U/ml) strongly inhibited I_{Cl} activated by Iso (0.1 μmol/L, Fig 2A). The time course of changes in relative current at +78 mV is shown in Fig 2B. The TNF- inhibition of the Iso-induced I_{Cl} occurred within

Fig4. Effect of $\text{TNF-}\alpha$ on FK-induced I_{Cl} . (A) The superimposed current traces recorded at control, $1\text{ }\mu\text{mol/L}$ FK, $1\text{ }\mu\text{mol/L}$ FK plus $1,000\text{ U/ml}$ $\text{TNF-}\alpha$ are shown. The horizontal bar indicates the zero current level. (B) For each trial, I_{Cl} at $+78\text{ mV}$ was normalized to the maximum level recorded during that trial. The relative currents were plotted against time, and the horizontal bars above the graph indicate extracellular application of FK ($1\text{ }\mu\text{mol/L}$) and $\text{TNF-}\alpha$ ($1,000\text{ U/ml}$).

2–5 min (Fig 2B).

The $\text{TNF-}\alpha$ sensitive current was obtained as the difference between the Iso-induced I_{Cl} recorded in the absence and presence of $\text{TNF-}\alpha$. The I–V curve of the $\text{TNF-}\alpha$ sensitive current had a weak outward-rectifying property and crossed at around -27 mV (Fig 2C), which is also near the equilibrium potential for Cl^- (-30 mV). Thus, this change could be attributable to I_{Cl} . Similar results were obtained in 11 other cells and the averaged reversal potential was $-25.4 \pm 0.7\text{ mV}$ ($n=11$). Ca^{2+} channel current inhibition by $\text{TNF-}\alpha$ was reported to recover after $\text{TNF-}\alpha$ washout¹³ but in the present experiment, I_{Cl} did not recover during washout for 3 min.

Concentration–Response Relationship for the Effects of $\text{TNF-}\alpha$ on Iso-Induced I_{Cl}

The concentration dependence of I_{Cl} inhibition by $\text{TNF-}\alpha$ was examined in the presence of $0.1\text{ }\mu\text{mol/L}$ Iso. The magnitude of the $\text{TNF-}\alpha$ effect was evaluated as the difference between the amplitude of I_{Cl} at $+78\text{ mV}$ in the absence and presence of $\text{TNF-}\alpha$ (Fig 3A). Representative data is shown in Fig 3A. $\text{TNF-}\alpha$ (300 U/ml) failed to inhibit I_{Cl} activated by Iso ($0.1\text{ }\mu\text{mol/L}$) completely. The time course of changes in the current amplitude is given in Fig 3B. The $\text{TNF-}\alpha$ inhibition of Iso-induced I_{Cl} reached the steady state (Fig 3B). In Fig 3C, the relative current is calculated as a function of $\text{TNF-}\alpha$ concentration. Each bar represents the result obtained from 5–10 cells. $\text{TNF-}\alpha$ decreased the amplitude of Iso-induced I_{Cl} in a concentration-dependent manner ($30\text{–}1,000\text{ U/ml}$, $n=30$). The concentration for the half-maximal effect (IC_{50}) was 144 U/ml .

Effects of $\text{TNF-}\alpha$ on Forskolin-Induced I_{Cl}

To determine whether or not $\text{TNF-}\alpha$ could interfere with

Fig5. Effect of PTX on the Iso-induced I_{Cl} inhibition by $\text{TNF-}\alpha$. Chart records are shown on the left and difference currents on the right. Vertical deflection shows the response to ramp pulses ($\pm 120\text{ mV}$, $\pm 0.46\text{ V/s}$), which were applied every 15 s from -40 mV of holding potential. The negative limb of the ramp pulse was used to plot the I–V curve.

the binding of Iso to α -adrenergic receptors, its effects on forskolin (FK), a direct activator of adenylate cyclase²⁵ was examined. Although the time course of the response to FK was slower than that to Iso, FK ($1\text{ }\mu\text{mol/L}$) activated I_{Cl} markedly. $\text{TNF-}\alpha$ ($1,000\text{ U/ml}$) suppressed the increase in I_{Cl} induced by FK ($n=5$) just as in Iso-induced I_{Cl} (Fig 4).

Effect of PTX on the I_{Cl} Inhibition by $\text{TNF-}\alpha$

The GTP binding regulatory proteins (G proteins) play a pivotal role in the transduction of various biological signals. Pertussis toxin (PTX) ADP-ribosylates a subunit of G_i and G_o proteins and inhibits their function²⁶. Because carbachol (CCh) is known to inhibit the I_{Cl} induced by Iso via PTX-sensitive G proteins^{24,27}, we investigated an association between the inhibitory action of $\text{TNF-}\alpha$ and PTX-sensitive G proteins.

After prior incubation with PTX ($5\text{ }\mu\text{g/ml}$, at 36°C , $>60\text{ min}$), the inhibitory effect of CCh was virtually absent. However, the effect of $\text{TNF-}\alpha$ was still observed even in PTX-treated cells, indicating less involvement of PTX sensitive G proteins (Fig 5).

Effects of $\text{TNF-}\alpha$ on I_{Cl} Induced by Increased Intracellular cAMP

The effect of $\text{TNF-}\alpha$ on I_{Cl} induced by an increase in intracellular cAMP was analyzed with the experimental protocol shown in Fig 6A. To assess whether the inhibitory action of $\text{TNF-}\alpha$ is mediated by reducing cAMP concentration, we performed the experiment under the condition in which intracellular cAMP concentration was clamped.

The increase in intracellular cAMP was produced by adding 8-bromo-cAMP ($100\text{ }\mu\text{mol/L}$) to the internal solution, and IBMX (0.1 mmol/L) to the external solution. As shown in Fig 6A, a large outwardly rectifying current was observed soon after establishing the whole-cell condition. After replacement of the standard external solution with the low Cl^- external solution, the current amplitude was reduced and the effect was clear at more positive membrane potentials (Fig 6A). This result indicated that the current induced by the increase in cAMP might be carried mainly by Cl^- . The superfusate was then changed to the standard

Fig 6. Effect of TNF- α on cAMP loaded cells. (A) Chart recording of the membrane currents. Vertical deflection shows the response to ramp pulses (± 120 mV, ± 0.46 V/s) which were applied every 15 s from the holding potential of -40 mV. The horizontal bars at the top indicate internal dialysis with 8-bromo-cAMP ($100 \mu\text{mol/L}$), extracellular application of iso-butylmethylxanthine (IBMX, 0.1 mmol/L), TNF- α ($1,000 \text{ U/ml}$), and change of the external Cl^- concentration to 45 mmol/L . Arrows labeled a, b and c corresponds to those in (B). (B) I-V curve relations measured by ramp pulses as in (A). The negative limb of the ramp pulse was used to plot the I-V curve.

external solution and TNF- α ($1,000 \text{ U/ml}$) was applied; however, it failed to inhibit the I_{Cl} (Fig 6).

Effect of TNF- α on the Iso-Induced I_{Cl} in the Presence of a NOS Inhibitor

TNF- α is known to activate NOS and it has been recently reported that NO antagonized the α -adrenergic action in cardiac myocytes.²⁸⁻³¹ To test the hypothesis that TNF- α -activated NO production diminishes α -adrenergic responses, we examined the effect of pretreatment with L-NAME, a specific inhibitory compound of NOS.

L-NAME (0.1 mmol/L) was added in the external solution at least 20 min before recording the control currents. Ramp pulses (± 120 mV, -0.4 V/s) were applied from -40 mV of the holding potential every 15 s. To rule out the possibility that L-NAME might inhibit I_{Cl} , we first examined the effect of the absence and presence of L-NAME (0.1 mmol/L) on I_{Cl} . As summarized in Fig 7, Iso ($0.1 \mu\text{mol/L}$) enhanced I_{Cl} , regardless of the presence of L-NAME, and there was no significant difference in the response to Iso ($0.1 \mu\text{mol/L}$), indicating that L-NAME did not interfere with the adrenergic action of Iso on I_{Cl} . We then assessed the effect of TNF- α while NOS was inhibited by L-NAME (0.1 mmol/L). As shown in Fig 7, TNF- α significantly decreased the magnitude of Iso-induced I_{Cl} despite the presence of L-NAME.

Discussion

The function of a wide variety of cardiac ion channels can be modulated by α -adrenergic stimulation, which contributes to changes in pacing and the contractile activity of the heart. In various pathological conditions, TNF- α evokes

Fig 7. Effect of NOS inhibitor on the Iso-induced I_{Cl} inhibition by TNF- α . The coordinate axis of the histogram shows the Iso-induced relative currents at ± 78 mV after exposure to standard external solution (control), L-NAME (0.1 mmol/L), TNF- α ($1,000 \text{ U/ml}$), and L-NAME (0.1 mmol/L) plus TNF- α ($1,000 \text{ U/ml}$). Data are expressed as means $\pm$ standard error of the mean of $n=5-11$ wells. * $p < 0.005$ in comparison with control values using Student's unpaired t-test. ** $p < 0.005$ in comparison with relative current measured in the presence of Iso ($0.1 \mu\text{mol/L}$) plus L-NAME (0.1 mmol/L) using Student's unpaired t-test.

hemodynamic abnormalities; however, its influence on the α -adrenergic response is still unclear. In the present study, we demonstrated that TNF- α rapidly antagonizes the α -adrenergic responses of I_{Cl} .

Previous studies have implicated α -adrenergic hypo-responsiveness as a mechanism of the cardiodepressant effect of TNF- α .³²⁻³⁴ Gulick et al have reported that 100 U/ml human recombinant TNF- α inhibits the Iso ($0.1 \mu\text{mol/L}$) stimulated cAMP formation by 40% in cultured neonatal rat cardiac myocytes.³² That effect was not immediate, but required several hours to become apparent. In the present study, I_{Cl} inhibition occurred within 2–5 min after TNF- α exposure (Fig 2B). We observed that TNF- α reversed the increase in I_{Cl} induced by Iso (Fig 2A). As shown in Fig 3, the potency of TNF- α for inhibiting Iso ($0.1 \mu\text{mol/L}$) induced I_{Cl} , given as an IC_{50} value, was 144 U/ml , which was almost identical to the value reported by Gulic et al.³² However, the time course for expression of the anti-adrenergic action in the present study was much earlier than that previously reported. The I_{Cl} inhibition occurred within 2–5 min of TNF- α exposure (Fig 2B), which indicates that TNF- α rapidly (within several minutes) exerts an anti-adrenergic action in adult guinea-pig ventricular myocytes. A possible reason for the discrepancy between the previous results³² and ours might be an age difference because the anti-adrenergic action was assessed in neonatal cardiac myocytes³² and in adult ventricular myocytes (present study). During development, modulatory responses to catecholamines can be affected by changes in the relative expression levels of the α -adrenergic signaling cascade component.³⁵

Enhancement of I_{Cl} can be caused by a α -adrenergic receptor-mediated cAMP-dependent phosphorylation of the Cl^- channel. To elucidate whether the inhibitory action of TNF- α was exerted by a modulation of the cAMP-dependent phosphorylation pathway, pharmacological tools such as FK, 8-bromo-cAMP and IBMX, were used. As shown in Fig 4, TNF- α reversed FK-induced I_{Cl} , indicating that the inhibitory action does not involve the signaling cascade upstream from adenylate cyclase. Moreover, TNF- α was unable to inhibit I_{Cl} induced by 8-bromo-cAMP and

IBMX (Fig 6). The results suggest that the inhibitory action of TNF- α is not caused by the direct modulation of the Cl⁻ channel, but occurs by the inhibition of adenylate cyclase and/or reducing intracellular cAMP concentration.

The effect of α -adrenergic agonists is antagonized with muscarinic agonists and it is accepted that the activation of adenylate cyclase by G_s protein is inhibited by the muscarinic receptor stimulation through PTX-sensitive G_i protein. A previous report has indicated that a 72-h exposure of cardiac myocytes to immune cell cytokines inhibits cAMP accumulation elicited by α -adrenergic agonist via PTX-sensitive G proteins.³³ As shown in Figs 2 and 4, the anti-adrenergic action of TNF- α could be analogous to that of muscarinic agonists. However, prior exposure to PTX did not affect the inhibitory action of TNF- α on Iso-induced I_{Cl} (Fig 5). These results suggest that the rapid anti-adrenergic action of TNF- α is not mediated via the PTX-sensitive G proteins regulatory pathway. Moreover, TNF- α was unable to inhibit I_{Cl} induced by 8-bromo-cAMP and IBMX (Fig 6). These results also suggest that the inhibitory action of TNF- α is not through direct modulation of Cl⁻ channel, but occurs by the inhibition of adenylate cyclase and/or reducing intracellular cAMP concentration.

Another controversial issue has been the relative importance of NOS to the anti-adrenergic action of TNF- α . TNF- α is known to increase the generation of NO in a number of different tissues and cell types, including cardiac myocytes, in vivo and in vitro.^{36,37} Several reports have suggested that NO plays an essential role in muscarinic inhibition of Ca²⁺ current augmented by α -adrenergic stimulation in rabbit nodal cells.^{27,28,38} Balligand et al have postulated that the activation of an endogenous NOS appears to modulate cardiac myocyte responsiveness to muscarinic cholinergic and α -adrenergic receptor stimulation in cultured neonatal and adult rat cells.³⁸ On the other hand, it has also been reported that NOS activity in guinea-pig ventricular myocytes is not involved in muscarinic inhibition of cAMP-regulated ion channels.³⁹ As shown in the present study, TNF- α could reverse the Iso-induced increase in I_{Cl} despite the presence of L-NAME (Fig 7), and the inhibitory effect of TNF- α was commensurate with that in the absence of L-NAME. Moreover, ventricular cells were dialyzed with a relatively high concentration of EGTA (5 mmol/L), which might limit any response involving a Ca²⁺-dependent mechanism that regulates the cNOS isozymes activity.⁴⁰ These results suggest that cNOS activation is less relevant to the I_{Cl} inhibition by TNF- α .

In conclusion, the present data suggest that TNF- α rapidly antagonizes the increase in I_{Cl} induced by α -adrenergic stimulation, and that the anti-adrenergic action is mediated by reducing cAMP concentration, probably by inhibiting adenylate cyclase activity, but not by the PTX-sensitive G proteins regulatory pathway or cNOS activation. A further direction of this study will be to clarify how TNF- α reduces cAMP concentration. In addition, the possible involvement of sphingosines in the antiadrenergic action of TNF- α should be addressed.

TNF- α is considered to be a double facet cytokine that has beneficial or deleterious effects during heart failure.⁴¹ TNF- α may be expected to prevent the cardiotoxicity of catecholamines or counteract the positive inotropic effects of adrenergic agonists.

Acknowledgments

We thank Dr Kyoichi Ono for critically reading the manuscript, and Katsuhisa Ikeda, Susumu Fujisawa and Dr Ichirota Nakajima for their technical support in carrying out the experiments.

References

1. Old LJ. Tumor necrosis factor. *Science* 1985; **230**: 630–632.
2. Torre-Amione G, Kapadia S, Lee J, Bies RD, Lebovitz R, Mann DL. Expression functional significance of tumor necrosis factor receptors in human myocardium. *Circulation* 1995; **92**: 1487–1493.
3. Kapadia S, Lee J, Torre-Amione G, Birdsall HH, Ma TS, Mann DL. Tumor necrosis factor- α gene and protein expression in adult feline myocardium after endotoxine administration. *J Clin Invest* 1995; **96**: 1042–1052.
4. Matsumori A, Yamada T, Suzuki H, Matoba Y, Sasayama S. Increased circulating cytokines in patients with myocarditis and cardiomyopathy. *Br Heart J* 1994; **72**: 561–566.
5. Maury CPJ, Teppo AM. Circulating tumor necrosis factor- α (cachectin) in myocardial infarction. *J Intern Med* 1989; **225**: 333–336.
6. Kumar BA, Thota V, Dee L, Olson J, Uretz E, Parrillo JE. Tumor necrosis factor and interleukin 1 are responsible for in vitro myocardial cell depression induced by human septic shock serum. *J Exp Med* 1996; **183**: 949–958.
7. Nagashima H, Kawashiro-Hirata N, Imamura K, Shimamoto K, Kawana M, Kasanuki H. Congestive heart failure after peripheral blood stem cell transplantation: Role of cytokines. *Jpn Circ J* 2000; **64**: 382–384.
8. Mann DL, Young JB. Basic mechanisms in congestive heart failure: Recognizing the role of proinflammatory cytokines. *Chest* 1994; **105**: 897–904.
9. Meldrum DR. Tumor necrosis factor in the heart. *Am J Physiol* 1998; **274** (Regulatory Integrative Comp Physiol **43**): R577–R595.
10. Ungureanu-Longrois D, Balligand JL, Kelly RA, Smith TW. Myocardial contractile dysfunction in the systemic inflammatory response syndrome: Role of a cytokine-inducible nitric oxide synthase in cardiac myocytes. *J Mol Cell Cardiol* 1995; **27**: 155–167.
11. Schulz R, Panas DL, Gatena R, Moncada S, Olley PM, Lopaschuk GD. The role of nitric oxide in cardiac depression induced by interleukin-1 and tumor necrosis factor- α . *Br J Pharmacol* 1995; **114**: 27–34.
12. Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hattler BG, Simmons RL. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* 1992; **257**: 387–389.
13. Krown KA, Yasui K, Brooker MJ, Dubin AE, Nguyen C, Harris GL, et al. TNF- α receptor expression in rat cardiac myocytes: TNF- α inhibition of L-type Ca²⁺ current and Ca²⁺ transients. *FEBS Lett* 1995; **376**: 24–30.
14. Oral H, Dorn GW, Mann DL. Sphingosine mediates the immediate negative inotropic effects of tumor necrosis factor- α in the adult mammalian cardiac myocyte. *J Biol Chem* 1997; **272**: 4836–4842.
15. Harvey RD, Hume JR. Autonomic regulation of a chloride current in heart. *Science* 1989; **244**: 983–985.
16. Bahinski A, Nairn AC, Greengard P, Gadsby DC. Chloride conductance regulated by cyclic AMP-dependent protein kinase in cardiac myocytes. *Nature* 1989; **340**: 718–721.
17. Tareen FM, Ono K, Noma A, Ehara T. α -adrenergic and muscarinic regulation of the chloride current in guinea-pig ventricular cells. *J Physiol* 1991; **440**: 225–241.
18. James AF, Xie LH, Fujitani Y, Hayashi S, Horie M. Inhibition of the cardiac protein kinase A-dependent chloride conductance by endothelin-1. *Nature* 1994; **370**: 297–300.
19. Isenberg G, Klöckner U. Glycocalyx is not required for slow inward calcium current in isolated rat heart myocytes. *Nature* 1980; **284**: 358–360.
20. Nakajima I, Watanabe H, Iino K, Saito T, Miura M. Ca²⁺ overload evokes a transient outward current in guinea-pig ventricular myocytes. *Circ J* 2002; **66**: 87–92.
21. Taniguchi J, Kokubun S, Noma A, Irisawa H. Spontaneously active cells isolated from the sino-atrial and atrio-ventricular nodes of the rabbit heart. *Jpn J Physiol* 1981; **31**: 547–558.
22. Hamil OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 1981; **391**: 85–100.
23. Zakharov SI, Harvey RD. Altered α -adrenergic and muscarinic response of CFTR Cl⁻ current in dialyzed cardiac myocytes. *Am J Physiol* 1995; **268** (Heart Circ Physiol **37**): H1795–H1802.

24. Horie M, Hwang TC, Gadsby DC. Pipette GTP is essential for receptor-mediated regulation of Cl^- current in dialysed myocytes from guinea-pig ventricle. *J Physiol* 1992; **455**: 235–246.
25. Seamon KB, Padgett W, Daly JW. Forskolin: Unique diterpene activator of adenylate cyclase in membranes and intact cells. *Proc Natl Acad Sci USA* 1981; **78**: 3363–3367.
26. Katada T, Ui M. Islet-activating protein: A modifier of receptor-mediated regulation of rat islet adenylate cyclase. *J Biol Chem* 1981; **256**: 8310–8317.
27. Zakharov SI, Harvey RD. Rebound stimulation of the cAMP-regulated Cl^- current by acetylcholine in guinea-pig ventricular myocytes. *J Physiol* 1997; **499**: 105–120.
28. Wahler GM, Dollinger SJ. Nitric oxide donor SIN-1 inhibits mammalian cardiac calcium current through cGMP-dependent protein kinase. *Am J Physiol* 1995; **268** (*Cell Physiol* **37**): C45–C54.
29. Han X, Shimoni Y, Giles WR. A cellular mechanism for nitric oxide-mediated cholinergic control of mammalian heart rate. *J Gen Physiol* 1995; **106**: 45–65.
30. Yamamoto S, Tsutsui H, Tagawa H, Saito K, Takahashi M, Tada H, et al. Role of myocyte nitric oxide in β -adrenergic hypo-responsiveness in heart failure. *Circulation* 1997; **95**: 1111–1114.
31. Han X, Kobzik L, Balligand JL, Kelly RA, Smith TW. Nitric oxide synthase (NOS3)-mediated cholinergic modulation of Ca^{2+} current in adult rabbit atrioventricular nodal cells. *Circ Res* 1996; **78**: 998–1008.
32. Gulick T, Chung MK, Pieper SJ, Lange LG, Schreiner GF. Interleukin 1 and tumor necrosis factor inhibit cardiac myocyte β -adrenergic responsiveness. *Proc Natl Acad Sci USA* 1989; **86**: 6753–6757.
33. Chung MK, Gulick TS, Rotondo RE, Schreiner GF, Lange LG. Mechanism of cytokine inhibition of β -adrenergic agonist stimulation of cyclic AMP in rat cardiac myocytes: Impairment of signal transduction. *Circ Res* 1990; **67**: 753–763.
34. Balligand JL, Ungureanu D, Kelly RA, Kobzik L, Pimental D, Michel T, et al. Abnormal contractile function due to induction of nitric oxide synthesis in rat cardiac myocytes follows exposure to activated macrophage-conditioned medium. *J Clin Invest* 1993; **91**: 2314–2319.
35. An RH, Davies MP, Doevendans PA, Kubalak SW, Bangalore R, Chien KR, et al. Developmental changes in β -adrenergic modulation of L-type Ca^{2+} channels in embryonic mouse heart. *Circ Res* 1996; **78**: 371–378.
36. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: Physiology, pathology and pharmacology. *Pharmacol Rev* 1991; **43**: 109–140.
37. Kelly RA, Balligand JL, Smith TW. Nitric oxide and cardiac function. *Circ Res* 1996; **79**: 363–380.
38. Balligand JL, Kelly RA, Marsden PA, Smith TW, Michel T. Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. *Proc Natl Acad Sci USA* 1993; **90**: 347–351.
39. Zakharov SI, Pieramici S, Kumar GK, Prabhakar NR, Harvey RD. Nitric oxide synthase activity in guinea pig ventricular myocytes is not involved in muscarinic inhibition of cAMP-regulated ion channels. *Circ Res* 1996; **78**: 925–935.
40. Su Z, Blazing MA, Fan D, George SE. The calmodulin-nitric oxide synthase interaction: Critical role of the calmodulin latch domain in enzyme activation. *J Biol Chem* 1995; **270**: 29117–29122.
41. Ferrari R. Tumor necrosis factor in CHF: A double facet cytokine. *Cardiovasc Res* 1998; **37**: 554–559.