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Tetrandrine ameliorates ischaemia-reperfusion injury of rat myocardium through inhibition of neutrophil priming and activation

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1 We have previously shown that tetrandrine (TTD), a bisbenzyltetrahydroiosquinoline isolated from the Chinese herb *Stephania tetrandra*, inhibits neutrophil adhesion, Mac-1 expression, and reactive oxygen species (ROS) production. To examine whether inhibition of neutrophil function may confer upon TTD the ability to prevent myocardial ischaemia-reperfusion (MI/R) injury, experiments were performed on rats subjected to coronary ligation followed by reperfusion for induction of MI/R injury.

2 Intravenous administration of TTD (0.1 and 1.0 mg kg^{-1}) 15 min prior to coronary ligation completely prevented MI/R-associated mortality. TTD pretreatment also significantly reduced MI/R-induced ventricular tachyarrhythmia, myocardial infarct size, and neutrophil infiltration.

3 However, TTD pretreatment did not influence mean arterial blood pressure, heart rate, or product of pressure-rate, indicating that TTD extenuated MI/R through mechanisms independent of modulating haemodynamics or myocardial oxygen demand.

4 Peripheral blood neutrophils were isolated for *ex vivo* examination of shape change and Mac-1 upregulation of neutrophils, two sensitive indicators of proinflammatory priming, as well as *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced adhesion and ROS production, parameters commonly used for the assessment of neutrophil activation.

5 Neutrophils from MI/R animals showed significant shape change and Mac-1 upregulation, both of which were prevented by TTD-pretreatments. On the other hand, fMLP-induced adhesion and ROS production of neutrophils were markedly enhanced by MI/R but diminished in TTD-pretreated animals.

6 These data suggest that the protective effect of TTD against MI/R injury can be accounted for by inhibition of neutrophil priming and activation, thereby abolishing subsequent infiltration and ROS production that cause MI/R injury.

Keywords: Adhesion; arrhythmia; Mac-1 upregulation; myocardial ischaemia-reperfusion injury; myocardium infarction; neutrophils; priming; reactive oxygen species; shape change; tetrandrine

Abbreviations: BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescencein acetoxymethyl ester; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescin; EB, ethidium bromide; FBS, foetal bovine serum; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FSC, forward scatter; HBSS, Hanks' balanced salt solution; HE, hydroethidine; H_2O_2, hydrogen peroxide; LMCA, left main coronary artery; Mac-1, macrophage adhesion molecule-1, also, CD11b/CD18; MI/R, myocardial ischaemia-reperfusion; $O_2^{\bullet -}$, superoxide anion; PBS, phosphate-buffered saline; PPB, potassium phosphate buffer; PRI, pressure-rate index; ROS, reactive oxygen species; SSC, side scatter; TTD, tetrandrine

Introduction

Tetrandrine (TTD) is a bisbenzyltetrahydroisoquinoline alkaloid isolated from the Chinese medicinal herb 'Hanfangji' (*Stephania tetrandra* S Moore) (Huang & Hong, 1998). It has been shown to be beneficial in various cardiovascular diseases, such as hypertension (Chen *et al*., 1991), arrhythmia (Cha *et al*., 1981), angina (Yu, 1986; Zhang & Liu, 1983), and pulmonary and portal hypertension (Huang *et al*., 1997). TTD can also protect animals from ischaemia and reperfusion-induced myocardial injury (Yao *et al*., 1995; Yu, 1983); however, the mechanisms by which TTD prevents such injury remain unclear.

Myocardial ischaemia-reperfusion (MI/R) can induce lethal ventricular arrhythmia and myocardial infarction (Lazzara *et al*., 1978; Parratt, 1994). Contributing factors to the induction of MI/R injury include generation of oxygen free radicals (Ferrari *et al*., 1991), overload of calcium (Liu *et al*., 1993), and infiltration of neutrophils (Mullane *et al*., 1984; Wysocki, 1992). The involvement of neutrophils in the development of MI/R injury has long been emphasized because neutrophils preferentially accumulate in the subendocardium during ischaemia (Go *et al*., 1988) and the accumulation is markedly accelerated following reperfusion (Engler *et al*., 1986). Furthermore, it has been shown that myocardial infarct size could also be reduced by systemic administration of antiserum to neutrophils (Jolly *et al*., 1986; Romson *et al*., 1983) or by decreasing the number of peripheral neutrophils (Litt *et al*., 1989). These observations establish the crucial role of neutrophil infiltration in causing myocardial damage following MI/R.

A prerequisite for neutrophil-mediated tissue damage is the 'priming' effect of various proinflammatory stimuli generated

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by damaged tissue during MI/R; these include lipid mediators (e.g., platelet activating factor [PAF]), cytokines (e.g., tumour necrosis factor-α [TNF-α]), and bacterial products (e.g., *N*-formyl-methionyl-leucyl-phenylalanine [fMLP]) (Kitchen *et al*., 1996). These agonists can elicit neutrophil polarization (i.e., shape change), up-regulation of cell-surface glycoproteins (e.g., fMLP receptor and Mac-1), as well as enhanced responses (e.g., adhesion, ROS production, and degranulation) to other stimulants (Pabst, 1994; Williams *et al*., 1990). Therefore, primed neutrophils are more likely to adhere to endothelium during MI/R and, subsequently, infiltrate into ischaemic lesions (Albelda *et al*., 1994), where they become 'activated' to release hydrolytic enzymes and large amounts of ROS that result in further damage (Lucchesi & Mullane, 1986; Williams, 1994). In this postulated migration process of neutrophils, adhesion of these cells to the endothelium or extracellular matrix represents one of the key elements. The importance of neutrophil adhesion in the pathogenesis of MI/R injury can be illustrated by the observation that Mac-1 (CD11b/CD18) expression, the major subtype of integrins responsible for the firm adhesion of neutrophils to endothelium, is elevated during reperfusion as compared with levels measured prior to ischaemia (Dreyer *et al*., 1989). Pretreatment of neutrophils with monoclonal antibody to CD18 inhibits not only their adhesion to myocytes but also the generation of hydrogen peroxide by these cells (Entman *et al*., 1990). With its anti-adhesive and ROS scavenging capability (Shen *et al*., 1999), TTD could be of particular use as a protective agent against MI/R injury because it may block neutrophil infiltration and ROS production during the early stages of MI/R.

We have previously shown that TTD could inhibit Mac-1 expression, ROS production and neutrophil adhesion (Shen *et al*., 1999), functions representing priming and activation of neutrophils that are involved in the development of reperfusion injury (Bagchi *et al*., 1997). We hypothesize that the myocardial-protective effect of TTD may be mediated by interfering with these functional profiles of the neutrophil. The purpose of this study was, therefore, to examine whether TTD could moderate MI/R-induced arrhythmia and myocardial infarction through suppressing neutrophil function. To accomplish the investigation, we used coronary ligation-induced MI/R injury in the rat as a model system. In particular, we examined shape change, Mac-1 upregulation, and fMLP-induced adhesion and ROS production of neutrophils as indicators of priming and activation.

Methods

Animal preparation

Male Sprague-Dawley rats (250–300 g) were anaesthetized with urethane 1.25 g kg^{-1} administered intraperitoneally. The trachea was cannulated for artificial respiration and the jugular vein was cannulated for drug administration. The carotid artery was cannulated for monitoring the systemic blood pressure and heart rate through a Statham P23 XL transducer to display on a Gould RS-3200 Physiological Recorder (Gould, Cleveland, OH, U.S.A.). A standard lead-I electrocardiogram (ECG) was recorded by attaching silver electrodes to the extremities of the animals. Heparin (10 units ml^{-1} in 0.9% saline) was used only for flushing the arterial line. The chest was opened by a left thoracotomy followed by sectioning the fourth and fifth ribs, approximately 2 mm to the left border of the sternum. Positive pressure artificial respiration was initiated immediately with room air, using a tidal volume of 15 ml kg^{-1} of body weight at a rate of 50 strokes min^{-1}. Fifteen rats were used for each group to study the effect of TTD on ischaemia and reperfusion-induced arrhythmia. Peripheral neutrophils were collected for *ex vivo* studies of priming and activation of these cells. Animals were handled in accordance with a protocol approved by the Advisory Committee of Animal Center at National Yang-Ming University.

Induction of MI/R injury

To induce myocardial ischaemia *in vivo*, ligation of the coronary artery was applied using the method of Nossuli *et al*. (1997). After the anterior pericardium was incised, the heart was eased out of the chest by gently applying pressure on the rib cage. A 6-0 silk ligature was placed around the left main coronary artery (LMCA). A small plastic snare formed from a Portex P-270 cannula was threaded through the ligature and placed in contact with the heart. After completion of all surgical procedures, the heart was repositioned in the chest and the animal was allowed to stabilize for 15 min before baseline readings of ECG, heart rate (HR), and mean arterial blood pressure (MABP) were recorded. The pressure-rate index (PRI), a parameter used as an index of myocardial oxygen demand (Nossuli *et al*., 1997) was also calculated as the product of MABP + HR/1000. Animals that developed arrhythmia before recording was stabilized or a sustained hypotension to less than 70 mmHg during the procedures were discarded.

For the induction of MI/R injury, the protocol of Bani *et al*. (1998) was followed with modification. Briefly, the previously placed reversible silk ligature around the LMCA was tightened such that the vessel was completely occluded. This was designated at time '0'. Thirty minutes later, the LMCA ligature was unfastened and the ischaemic myocardium was allowed to reperfuse for an additional 60 min. Successful ligation of the coronary artery was validated by the observation of a decrease in arterial pressure and ECG changes (increased amplitude in R wave and elevation of ST segment), indicative of myocardial ischaemia. Reperfusion was confirmed by an increase in arterial pressure, ECG changes (decreased amplitude in R wave and normalization of ST segment) and sudden occurrence of arrhythmia. Experimental grouping of animals for this study included one sham-operated (Sham) group, two TTD-pretreated groups (MI/R + TTD), and one MI/R (MI/R only) group. In the 'Sham' group, rats were injected with 0.3 ml of saline instead of TTD and underwent the same surgical procedures as above without tightening the silk ligature around the LMCA. Whole blood from each group was drawn from the carotid artery immediately before coronary ligation and by cardiac puncture immediately after reperfusion into tubes containing heparin (10 unit ml^{-1}) for *ex vivo* studies of peripheral neutrophils.

Drug administration

Tetrandrine, purchased from Aldrich (Milwaukee, WI, U.S.A.), was dissolved in 1 N HCl and adjusted to pH 7.4 with 5 N NaOH to achieve a stock concentration of 10 mg ml^{-1}. Serial dilution in normal saline to adequate concentrations was performed from the stock immediately before use. TTD solution of 0.3 ml was administered at two different doses (to reach final dosages of 0.1 and 1.0 mg kg^{-1}) *via* intravenous injection 15 min before coronary occlusion. Normal saline (0.3 ml) was used as vehicle control in both

'Sham' and 'MI/R only' groups. Animals were randomly allocated to drug treatments (TTD, 0.1 and 1.0 mg kg^{-1}) or vehicle control.

Evaluation of arrhythmia, ischaemia and infarction of the myocardium

Before and during the period of myocardial ischaemia (30 min occlusion of coronary artery) and reperfusion (60 min reperfusion following occlusion), recordings of HR, MABP, and electrocardiogram (ECG) changes were performed using a personal computer equipped with a wave form data acquisition/analysis software (AcqKnowledge, Biopac System, Goleta, California, U.S.A.). Ventricular ectopic activity was evaluated according to the diagnostic criteria advocated in the Lambeth Convention (Walker *et al*., 1988): the number of ventricular premature beat (VPB) and the incidence and duration of ventricular tachyarrhythmias, including ventricular tachycardia (VT) and ventricular fibrillation (VF), in surviving animals were thus determined. Mortality was recorded and the rate was calculated.

Quantification of ischaemic and infarct zones was performed following Johnston *et al*. (1983). Animals were sacrificed after the end of reperfusion and hearts were irrigated through the great vessels with Kreb's solution at 37°C, 100 mmHg for 5 min to remove blood. Two ml of cardiogreen dye (1.0 mg ml^{-1} indocyanine green in Krebs solution) was then injected into the cardiac chambers to differentiate the non-ischaemic (green) from ischaemic (not stained) myocardium. The ischaemic zone was excised and weighed for the calculation of the ischaemic zone as a percentage of total ventricular weight. The ischaemic zone was then sliced into 1.0 mm sections and incubated with 10 mg ml^{-1} of 2,3,5-triphenyltetrazolium chloride (Sigma), pH 8.5, at 37°C for 40 min. Sections were fixed in 10% formaldehyde for 2 days before infarct (white) tissue manifested. The infarct zones were excised and weighed. The quantity of the infarct zone was normalized and expressed as a percentage of the weight of the ischaemic zone.

Evaluation of neutrophil infiltration in the myocardium

Myeloperoxidase (MPO) activity has been used as a quantitative assessment of neutrophil infiltration into ischaemic myocardium (Mullane *et al*., 1985). In this study, MPO activity was evaluated according to Bani *et al*. (1998) with modification. Briefly, frozen samples of ventricular tissue weighing approximately 100 mg were homogenized in 1.5 ml of potassium phosphate buffer (PPB, 50 mM, pH 6). One ml of the homogenate was centrifuged at $10{,}000 \times g$ for 10 min, and the pellet was suspended in 1 ml of PPB containing 0.5% hexadecyltrimethylammonium bromide (Sigma) to negate the pseudoperoxidase activity of haemoglobulin and to solubilize membrane-bound MPO. The suspensions were treated with three cycles of freezing and thawing, sonicated on ice for 10 s, and centrifuged at $12{,}000 \times g$ for 10 min. MPO activity was determined in the supernatants. Briefly, 0.1 ml of the supernatant was mixed with 2.9 ml of PPB containing 0.19 mg ml^{-1} of *o*-dianisidine chloride and 0.0005% H_2O_2 as a substrate for MPO. Oxidized *o*-dianisidine forms a soluble chromophore absorbing at wavelength of 460 nm and the absorbance (OD_{460}) was determined by spectrophotometry over 2 min. The values of tissue MPO activity were expressed as $OD_{460} \times 100$ mg^{-1} of proteins. Protein concentration was determined with a BCA kit (Pierce, Rockford, U.S.A.).

Determination of neutrophil shape changes by flow cytometry

Changes in forward scatter (FSC) and side (right angle) scatter (SSC) properties of neutrophils measured by a flow cytometric method have been reported to correlate with microscopically evaluated shape changes in stimulated human neutrophils (Cole *et al*., 1995). These changes in light scattering properties agree with changes in cell size, granularity, or both, which have been used as an indicator of neutrophil priming (Pabst, 1994; Kitchen *et al*., 1996). In this study, therefore, shape changes of neutrophils after MI/R were determined by flow cytometry. Briefly, whole blood was collected immediately before ligation (from the carotid artery) and after reperfusion (*via* cardiac puncture) from five randomly selected animals of each treatment group. Fifty μl of blood sample were incubated with 2 ml of lysing reagent (in mM): (NH_4Cl 155, EDTA 0.1, and $KHCO_3$ 10, pH 7.4) to lyse erythrocytes. After washing twice with PBS, the pellet was resuspended in flow cytometer sheath fluid (Becton Dickinson, Lincoln Park, NJ, U.S.A.) containing 1% paraformaldehyde. Samples were analysed immediately on a flow cytometer (FACSort®; Becton Dickinson). A total of 5000 neutrophils were collected with appropriate gating to assess the shape change. Data were expressed as the percentage of mean light scattering values of FSC or SSC after MI/R using mean values before ligation as 100%: Shape change (%) = [(mean values of FSC or SSC)$_{\text{post-MI/R}}$/(mean values of FSC or SSC)$_{\text{pre-ligation}}$] × 100.

Determination of Mac-1 upregulation of neutrophils by flow cytometry

Immediately after the end of reperfusion, whole blood was collected from five randomly selected rats of each group by cardiac puncture for the assessment of neutrophil Mac-1 expression. Direct immunofluorescent staining of Mac-1 was used following Hoffman & Hansen (1981) with modification. Briefly, 50 μl of whole blood were incubated with an aliquot of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (anti-rat CD11b, IgG_1; Serotec, Kidlington, Oxford, U.K.) for 30 min at 4°C in the dark. Two ml of lysing reagent (in mM): (NH_4Cl 155, EDTA 0.1, and $KHCO_3$ 10, pH 7.4) was added to lyse erythrocytes. After washing twice with PBS, the pellet was resuspended in flow cytometer sheath fluid (Becton Dickinson) containing 1% of paraformaldehyde. Samples were analysed immediately by FACSort. Neutrophils were gated to examine the surface Mac-1 expression by measuring the emissions at 525 nm (FL1) and data were expressed as peak channel fluorescence intensity.

Measurement of ROS production of neutrophils in response to fMLP stimulation

For *ex vivo* measurement of fMLP-induced production of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$) by neutrophils, the method of Haston *et al*. (1995) was followed with some modification. Briefly, 100 μl of whole blood from five randomly selected animals of each group was incubated at 37°C for 5 min with 1 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Inc., Eugene, OR, U.S.A.) and for an additional 15 min with 1 μM of hydroethidine (HE; Molecular Probes). The acetate moieties of DCFH-DA are

cleaved off intracellularly by esterases, liberating the membrane impermeable 2′,7′-dichlorofluorescin (DCFH) which fluoresces when oxidized to 2′,7′-dichlorofluorescein (DCF) by H_2O_2. Hydroethidine, on the other hand, is directly oxidized by $O_2^{\bullet-}$ to ethidium bromide (EB), which fluoresces intensely after intercalating with nucleic acids. After labelling, erythrocytes were lysed with 2 ml of lysing buffer (in mM): (NH_4Cl 155, $KHCO_3$ 10, EDTA 0.1, pH 7.4) and then stimulated with fMLP (1 μM) for 30 min. Neutrophils were gated for cytofluorometric analysis based on their light scattering, then the production of $O_2^{\bullet-}$ and H_2O_2 were detected by measuring emissions at 590 nm (FL2) and 525 nm (FL1) for EB ($O_2^{\bullet-}$) and DCF (H_2O_2), respectively, by FACSort. Data were expressed as peak channel fluorescence for each sample as described above.

Measurement of neutrophils adhesion in response to fMLP stimulation

The method by Vaporciyan *et al.* (1993) was used to measure *ex vivo* adhesion of peripheral neutrophils. Neutrophils were isolated from whole blood samples of five randomly selected animals of each group using dextran sedimentation and Ficoll-Hypaque centrifugation, followed by hypotonic lysis of residual erythrocytes as previously described (Shen *et al.*, 1999). Isolated neutrophils were then preloaded with 1 μM of 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescencein acetoxymethyl ester (BCECF-AM; Molecular Probes) and incubated for 30 min at 37°C, following which the cells were pelleted and washed twice with cold PBS, then resuspended in HBSS before use. Neutrophil adhesion was performed in flat-bottom 24-well tissue culture plates (Costar, Cambridge, MA, U.S.A.) precoated with fibrinogen. Two hundred μl per well of BCECF-labelled neutrophils (5×10^5 cells ml^{-1} in HBSS) was plated into individual wells. After stimulation with fMLP (1 μM) for 30 min at 37°C, non-adherent cells were removed by aspiration and the wells were gently washed twice with warm PBS. Adherent neutrophils were determined by measuring the fluorescence using the fluorescent plate reader (Cytofluor 2300, Millipore) with excitation at 485 nm and emission at 530 nm. Data were expressed as fluorescence intensity.

Statistical analysis

All values in the text and figures are presented as mean ± s.e.mean. Parametric data were analysed by analysis of variance (ANOVA) followed by *post-hoc* Dunnett's *t*-test for multiple comparison. Fisher exact tests were used to analyse the difference in the incidence of VT, VF, and mortality rate in a pair-wise manner. The changes in BP and HR (and the derived pressure-rate indices) among the four groups were analysed by repeated measures ANOVA.

Results

Effect of TTD on MABP, HR and PRI during the course of MI/R

Prevention of MI/R injury by TTD was examined in rats subjected to coronary ligation and reperfusion. It has been reported that TTD causes hypotension in the rat (Qian *et al.*, 1983). In this study, however, neither 0.1 nor 1.0 mg kg^{-1} of TTD pretreatment influenced the MABP or HR (expressed as pressure-rate index; PRI) (Figure 1) during the 90 min course of MI/R (repeated measures ANOVA, $P > 0.05$).

Effect of TTD on MI/R-induced arrhythmia and mortality

To further investigate the *in vivo* effect of TTD pretreatment on MI/R injury, incidence and duration of both VT and VF were individually recorded in each animal. Whereas no rats developed arrhythmia in the sham-operated group, MI/R resulted in a high incidence of VT (100%) and VF (73%) (Figure 2). Pretreatment with TTD (0.1 and 1.0 mg kg^{-1}) significantly reduced both the incidence (Fisher exact test, $P < 0.05$) and duration (Dunnett's test, $P < 0.05$) of VT and VF caused by MI/R. Furthermore, while MI/R caused 33% mortality (as compared to 0% in sham-operated group), TTD pretreatments completely prevented MI/R-induced mortality (Figure 2, right panel; Fisher exact test, $P < 0.05$).

Effect of TTD on MI/R-induced ischaemia, infarction and neutrophil infiltration

Extensive ischaemia and infarction, as measured by ischaemic zone (Figure 3, left panel) and infarct zone (Figure 3, middle panel), respectively, were observed following coronary ligation and reperfusion. Pretreatment of TTD significantly reduced the infarct size (Figure 3, middle panel; Dunnett's test, $P < 0.05$). The ischaemic zone was similar in all groups (Figure 3, left panel). MI/R also caused a marked increase in neutrophil infiltration to myocardium, as indicated by enhanced MPO activity ($OD_{460} \times 100$ mg^{-1} protein) in MI/R versus sham-operated heart tissues (Figure 3, right panel; Dunnett's test, $P < 0.05$). In TTD pretreated animals, MPO activity was significantly diminished (Dunnett's test, $P < 0.05$).

Effect of TTD on shape change of peripheral neutrophils induced by MI/R

MI/R injury could stimulate neutrophils to exhibit functional upheavals (Dreyer *et al.*, 1989). One of the enhanced

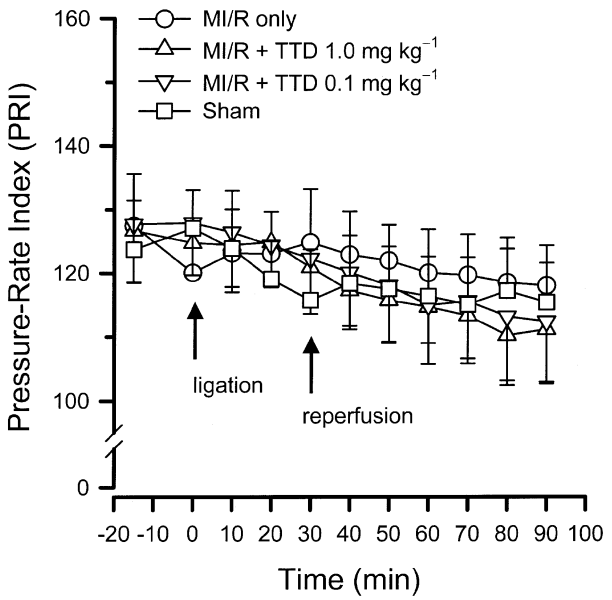


Figure 1 Effect of TTD on changes of pressure-rate index (PRI) in rats suffering from myocardial ischaemia-reperfusion. Rats subjected to MI/R injury in the absence or presence of TTD pretreatments (0.1 and 1.0 mg kg^{-1}) were monitored for MABP and HR. PRI was calculated as the product of MABP + HR/1000. Sham-operated animals were used as controls (Sham). Results are mean ± s.e.mean of $n = 10$ (for 'MI/R only') or 15 (for all other groups).

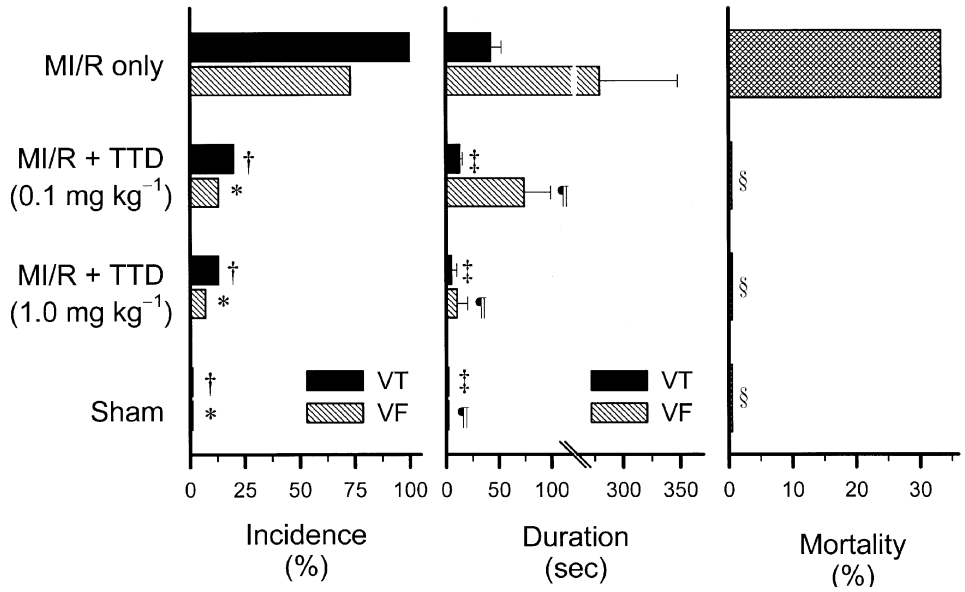


Figure 2 Effect of TTD on the incidence (left panel) and duration (middle panel) of arrhythmia and mortality (right panel) induced by MI/R. Rats were subjected to MI/R injury in the absence or presence of TTD pretreatment (0.1 and 1.0 mg kg^{-1}). Sham-operated animals without TTD pretreatment were included as controls (Sham). MI/R-induced arrhythmia, including ventricular tachycardia (VT) and fibrillation (VF) were analysed. Data of the duration of arrhythmia are expressed as mean ± s.e.mean of $n=10$ (for 'MI/R only' group) or 15 (for all other groups). For incidence of arrhythmia and mortality, $n=15$. *, †, §, $P<0.05$ by Fisher exact test and ¶, †, $P<0.05$ by Dunnett's test as compared with the 'MI/R only' group of respective experiments.

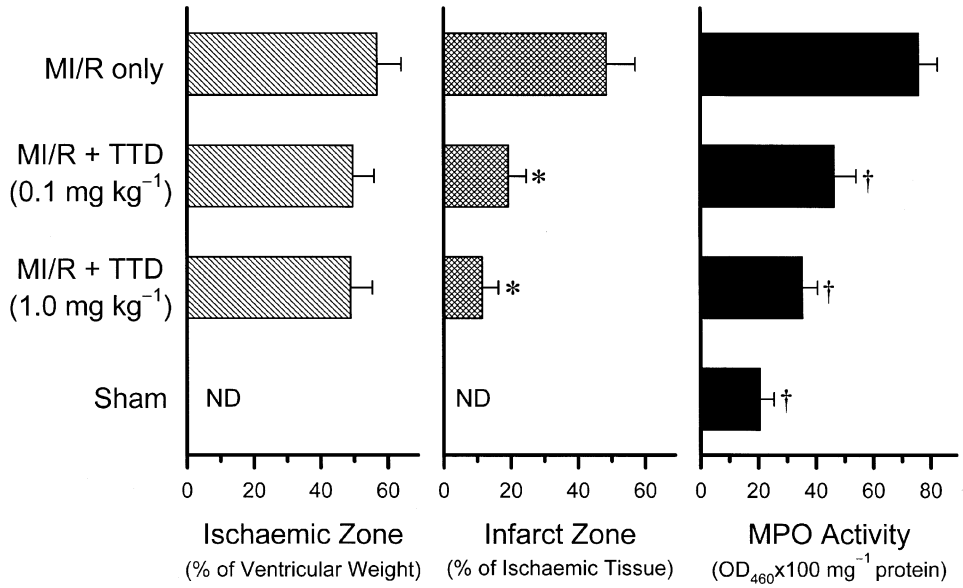


Figure 3 Effect of TTD on MI/R-induced ischaemia (expressed as ischaemic zone, left panel), infarction (expressed as infarct zone, middle panel), and neutrophil infiltration (measured as MPO activity, right panel) in the rat myocardium. Rats were subjected to MI/R injury in the absence or presence of TTD pretreatment (0.1 and 1.0 mg kg^{-1}). Sham-operated animals without TTD treatment were included as controls (Sham). Ischaemic and infarct zones were measured as per cent of weights of ventricles (1.12 ± 0.05 g) and ischaemic zone, respectively. Neutrophil infiltration in the myocardium was determined by MPO activities and data were expressed as mean ± s.e.mean of n-10 (for 'MI/R only' group) or 15 (for other groups). *, †, $P<0.05$ as compared with the MI/R only group of respective experiments using Dunnett's test. ND, not detected.

functions that represents a sensitive indicator of neutrophil priming is shape change, which can be assessed by forward and side (orthogonal) scattering properties of these cells using flow cytometry (Pabst, 1994; Kitchen *et al*., 1996). In this study, both forward scatter (FSC) and side scatter (SSC) properties were increased in neutrophils from MI/R animals (Figure 4a, middle panels) as compared to those from sham-operated group (Figure 4a, upper panels). Pretreatment with TTD (1.0 mg kg^{-1}) prevented such changes (Figure 4a, lower panels). A statistical summary of results from five experiments was presented in Figure 4b, illustrating that TTD pretreatment (1.0 mg kg^{-1}) significantly prevented MI/R-induced increases in FSC and SSC (Dunnett's test, $P<0.05$).

Effect of TTD on MI/R-induced Mac-1 upregulation in peripheral neutrophils

MI/R injury causes neutrophils to upregulate surface Mac-1 expression (Dreyer *et al*., 1989), another indicator of

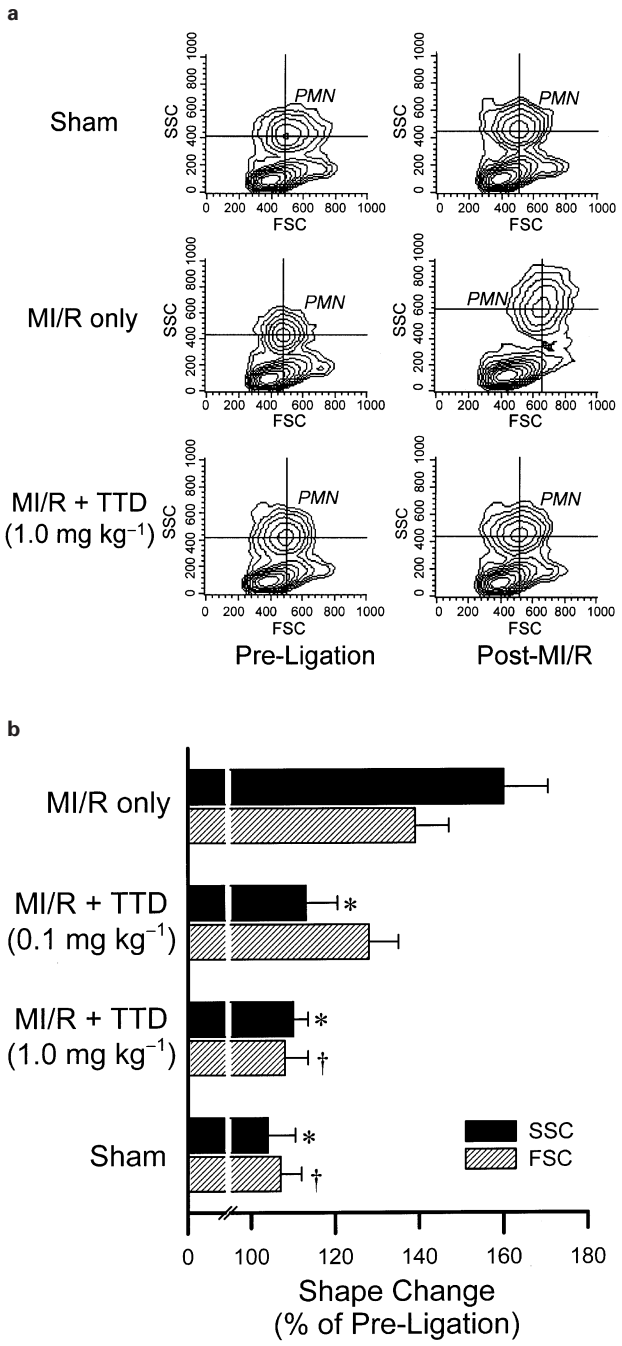


Figure 4 Effect of TTD on changes in light scattering properties (shape change) of neutrophils. Whole blood leucocytes were collected for the assessment of shape change by measuring forward scatter (FSC) and side (right angle) scatter (SSC) properties on a flow cytometer. (a) Representative contour plots of the output from a flow cytometric experiment. Neutrophils were designated as 'PMN'. (b) Statistical summary of TTD-inhibited neutrophil shape change. Percentage of pre-ligation values over post-MI/R values were calculated using the following formula: Shape change (%) = [(mean values of FSC or SSC)$_{\text{after MI/R}}$/(mean values of FSC or SSC)$_{\text{before ligation}}$] × 100. Data were expressed as mean ± s.e.mean of $n = 5$. *, †, $P < 0.05$ as compared to the 'MI/R only' group of respective parameters.

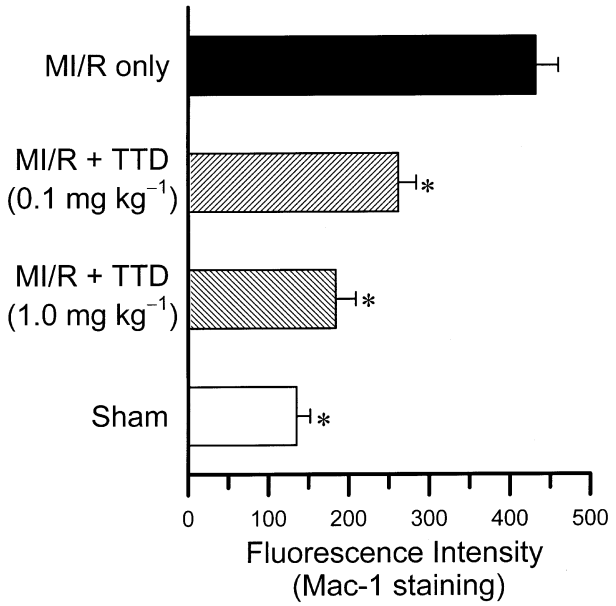


Figure 5 Effect of TTD on neutrophil surface Mac-1 upregulation induced by MI/R. Surface levels of Mac-1 were measured by staining with FITC-conjugated anti-rat CD11b and analysed on a FACSort® by gating neutrophils from the total leucocyte population. Data was expressed as peak channel fluorescence intensity and mean ± s.e.mean was calculated from five animals in each group. *, $P < 0.05$ as compared to the MI/R only group.

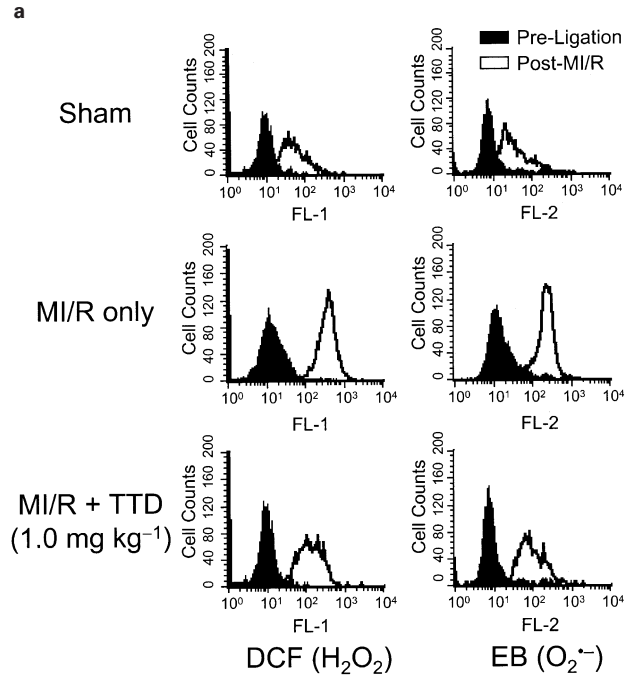
neutrophil priming (Pabst, 1994). Flow cytometric analysis of Mac-1 levels showed a 3.2 fold increase on neutrophils of the MI/R group, as compared to the sham-operated group (Figure 5; Dunnett's test, $P < 0.05$). On the other hand, TTD pretreatment (0.1 and 1.0 mg kg^{-1}) significantly inhibited MI/R-upregulated Mac-1 expression (Figure 5; Dunnett's test, $P < 0.05$).

Effect of TTD on fMLP-induced ROS production and adhesion of peripheral neutrophils following MI/R

Ischaemic and reperfused myocardium has been shown to generate chemotactic activity and subsequently activated neutrophil adhesion and production of proinflammatory mediators such as ROS (Dreyer *et al.*, 1989). To examine whether TTD could prevent neutrophils from being activated by MI/R, fMLP-induced ROS production (including H_2O_2 and $O_2^{\bullet -}$) and adhesion to fibrinogen were measured in these cells. MI/R-primed neutrophils produced significantly larger amounts of ROS than cells from sham group (Figure 6a,b; Dunnett's test, $P < 0.05$). In contrast, TTD pretreatments effectively inhibited such an increase in ROS production (Figure 6). On the other hand, while MI/R caused an enhancement in fMLP-stimulated adhesion of neutrophils up to 3 fold of sham-operated levels, TTD pretreatments markedly inhibited MI/R-enhanced neutrophil adhesion (Figure 7; Dunnett's test, $P < 0.05$).

Discussion

Infiltration of neutrophils to the infarct areas is a crucial process in the development of MI/R injury (Williams, 1994). This begins with enhanced adhesion of neutrophils to the endothelium, as primed and activated by proinflammatory stimuli generated from ischaemia (Pabst, 1994; Yoshida *et al.*, 1992), followed by the release of large amounts of ROS and proteolytic enzymes that mediate MI/R injury (Williams, 1994). Therefore, drugs that prevent neutrophils from priming (e.g., shape change and up-regulation of β2-integrins) and/or activation (e.g., adhesion and ROS



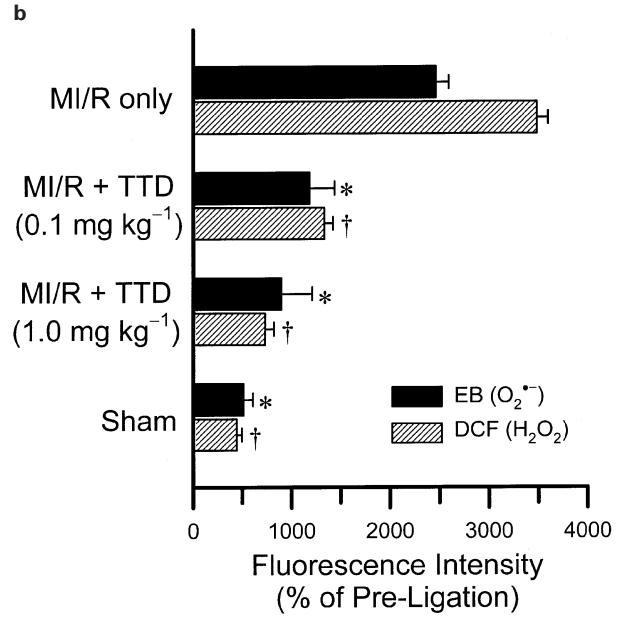


Figure 6 Effect of TTD on fMLP-induced ROS production by peripheral neutrophils. Whole blood (100 μl) collected from five rats in each group was incubated with 1 μM of HE (oxidized to EB by $O_2^{\bullet -}$) and DCFH-DA (oxidized to DCF by H_2O_2). After erythrocytes lysed, fMLP (1 μM) was added to induce ROS. Intracellular ROS production was determined on a flow cytometer by measuring the emissions at 525 nm, (FL1) for DCF (H_2O_2) and 590 nm (FL2) for EB ($O_2^{\bullet -}$). (a) Representative histograms of outputs from flow cytometric analysis. (b) Statistical summary of fMLP-induced ROS production from five experiments. Data are expressed as percentage of control and taking the values of respective sample from each group before ligation (pre-ligation) as 100%. *, † $P<0.05$ as compared to 'MI/R only' group of individual parameters.

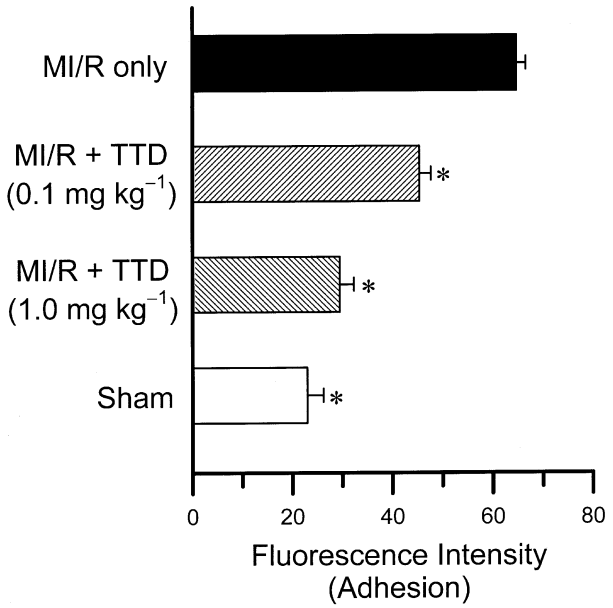


Figure 7 Effect of TTD on fMLP-induced adhesion of peripheral neutrophils. After MI/R, neutrophils were isolated by density centrifugation and loaded with BCECF-AM (1 μM). BCECF-labelled neutrophils (5×10^5) were plated on fibrinogen-coated 24-well plates and stimulated with fMLP (1 μM) for 30 min. Non-adherent cells were removed and adherent neutrophils were determined by measuring the fluorescence on a fluorescent plate reader. Data are expressed as fluorescence intensity. *$P<0.05$ as compared to 'MI/R only' group.

production) should prove effective as anti-ischaemic agents for the treatment of MI/R injury. In this study, we demonstrated that rats subjected to ligation of left coronary artery and subsequent reperfusion developed ventricular arrhythmia and myocardial infarction, both of which are known signs of MI/R injury (Lazzara *et al*., 1978; Song *et al*., 1996). If these animals were treated with 0.1 and 1.0 mg kg^{-1} (i.v.) of TTD 15 min prior to coronary ligation, the development of ventricular arrhythmia and myocardial infarction was significantly reduced. While comparable findings have been reported in previous studies (Yao *et al*., 1995), no mechanism was provided as to how TTD protects the heart from MI/R injury. We established in this study that TTD effectively prevented neutrophils from priming (as measured by neutrophil shape change and Mac-1 upregulation) and activation (as measured by ROS production and adhesion). Therefore, it is likely that the anti-ischaemic effect of TTD may be mediated by inhibiting neutrophil recruitment at the early stage, i.e., priming and activation.

TTD is beneficial in preventing animals from death while sustaining MI/R. All rats receiving TTD pretreatment survived MI/R, whereas 33% of animals subjected to MI/R without drug treatment died. Furthermore, the infarct size (measured as per cent weight of the total ischaemic zone) in the TTD-treated animals was significantly reduced. Parallel to this finding was the change in myocardial MPO activity, a biochemical marker of neutrophil infiltration, which was considerably increased following MI/R and reduced by TTD pretreatment. This indicates that inhibition of neutrophil infiltration may be responsible for the myocardium-protecting effect of TTD. The reduction in sizes of MI/R-induced infarct by TTD was not due to its influence on myocardial oxygen demand, since there was no significant difference in PRI, an index of oxygen demand (Nossuli *et al*., 1997), among all groups. Neither was the anti-ischaemic effect of TTD a result of modulation of general cardiovascular functions, as changes in MABP and HR during 90 min of MI/R period showed no difference among all groups. Taken together, these findings exclude the possibility that TTD protects the heart by altering

oxygen demand of the myocardium or MABP and HR during MI/R.

Infiltration of neutrophils to infarct areas and production of large amounts of ROS by these cells may play important roles in the development of MI/R injury (Williams, 1994). When the myocardium suffers ischaemia and reperfusion, local production of proinflammatory mediators, such as PAF, by the ischaemic tissue may alter the functional activity of neutrophils by priming them for subsequent activation in response to physiologic agonists (Puchnina-Artushenko *et al*., 1993; Stahl *et al*., 1988). Once primed, the shape of these neutrophils changes and the amount of surface adhesion molecules (such as Mac-1) increases (Miller *et al*., 1987), resulting in enhanced adhesion to endothelial cells (Haslett *et al*., 1985; Pabst, 1994). Adhesion to endothelium then promotes transmigration and infiltration of these neutrophils to myocardium, where they generate enormous quantities of ROS that aggravates the damage to the myocardium (Cohen, 1989). Since we have previously shown that TTD could inhibit neutrophil adhesion, Mac-1 upregulation and ROS production (Shen *et al*., 1999), we postulated that the MI/R prevention effect of TTD may rely on its capacity to inhibit neutrophils priming and activation and subsequent infiltration into infarct areas. Thus, we examined shape changes and surface Mac-1 upregulation of peripheral neutrophils immediately after MI/R, and the responses of these cells to fMLP-induced ROS production and adhesion. We found that neutrophils from MI/R-treated animals demonstrated marked shape change, upregulated Mac-1 expression, enhanced ROS production in response to fMLP, and increased adhesion to fibrinogen, all of which were diminished by TTD pretreatments. These results establish that neutrophils were primed by MI/R, and pretreatment of TTD suppressed neutrophil priming and activation.

Numerous pharmacological substances have been reported to decrease ischaemic damage or suppress ventricular arrhythmia in the ischaemic heart, such as calcium channel blockers (Wolfe *et al*., 1991), antioxidants (Hong *et al*., 1996; Lukovic *et al*., 1993), and nitric oxide donors (Wainwright & Martorana, 1993). Although depletion of neutrophils has been shown to alleviate arrhythmia (Kutsumi *et al*., 1989; Dhein *et al*., 1995), the association between the anti-neutrophil effect of these drugs and their anti-arrhythmic effect remains controversial. Since various pharmacological actions of TTD have been reported, mechanisms behind the antiarrhythmic and anti-ischaemic effects of TTD could be of multiple facets. For example, TTD could decrease the production of malonyldialdehyde, a lipid peroxidation metabolite in ischaemic/reperfused rat hearts (Ren *et al*., 1995). This suggests that TTD may inhibit peroxidation of cell membrane lipids by ROS generated during the MI/R, thus bestowing protection to cardiomyocytes that could be damaged by ROS. In addition, disturbed calcium homeostasis with excess calcium flux into cardiomyocytes is thought to be an important factor underlying irreversible myocardial injury (Bourdillon & Poole-Wilson, 1981). Because TTD could block the T- and L-type calcium channels in cardiomyocytes (Liu *et al*., 1992) and inhibit intracellular calcium rises in neutrophils (Shen *et al*., 1999), it is possible that the calcium antagonizing activity may mediate the anti-ischaemic and/or antiarrhythmic effects of TTD. Therefore, while our data suggest a correlation between the anti-neutrophil effect of TTD and its antiarrhythmic effect, further study is needed to elucidate the precise mechanisms for the preventative effects of TTD against MI/R-induced ventricular arrhythmias as well as myocardial damage.

In conclusion, we have shown that TTD is effective in protecting rats from ventricular arrhythmia, myocardial infarction, and death induced by MI/R. Modulation of MABP, HR, or myocardial oxygen demand by TTD does not mediate such protection. Instead, TTD protects the myocardium through inhibiting shape change and surface Mac-1 upregulation of neutrophils and, in turn, interfering with their infiltration to ischaemic tissues and subsequent generation of deleterious products that eventually lead to permanent injury of the myocardium. Our study supports that TTD or TTD-derived drugs may be of clinical usefulness in the prevention and treatment of ischaemic heart diseases.

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