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TETRACYCLINE PROTECTS MYOCARDIUM AGAINST ISCHEMIC INJURY

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1. ABSTRACT

Stress pretreatments protect myocardium from ischemic injury. We hypothesized that tetracycline, an antibiotic, may induce a stress response via the inhibition of mitochondrial translation as it induces the cold stress response by translational inhibition in *E. coli*. If so, tetracycline may protect myocardium from ischemic injury as stress pretreatments do. Thus, we investigated the effects of tetracycline on myocardial ischemia and its association with stress response. In a dog model of acute ischemia, 4mg/kg tetracycline injected 30 min prior to the occlusion improved the functional recovery from stunning of myocardium caused by ischemia. The same dosage of tetracycline dramatically reduced the size of infarct area in murine hearts analyzed by tetrazolium staining. In HeLa cells, tetracycline induced molecules that were increased by cold stress, which suggests that tetracycline may induce a cold stress-like response in mammalian cells. These molecules were also induced by ischemic stress in murine hearts, suggesting that the stress response caused by translational inhibition in mitochondria may be associated with the cardioprotection by tetracycline. Our results suggest that a subclinical dosage of tetracycline may protect heart from ischemic injury. Therefore, tetracycline may be of great use in suppressing the development of infarction caused by myocardial ischemia. This study is also important for providing new insights into the non-antimicrobial effects of tetracycline and its derivatives.

2. INTRODUCTION

The acute coronary syndromes are the leading cause for hospitalization and death among adults in industrialized countries. Myocardial protection by various stress-preconditioning against ischemia/reperfusion injury has been intensively investigated. In animal models, pretreatment of hearts by mild stresses has been known to induce cellular stress response and protect the heart from more severe ischemic injury (1, 2). Preconditioning of the heart by repeated ischemia and reperfusion has been reported to delay the onset of further irreversible injury (1, 3), reduce the subsequent post-ischemic ventricular dysfunction (4-7), and incidence of ventricular arrhythmia (3, 8). In addition to ischemic preconditioning, ventricular tachycardia (9, 10), whole body heat stress (11-13), and chronic hypoxia (14), have been reported to limit the myocardial infarct size in animal models.

The heat shock proteins (HSPs) have been shown to be induced in cardiac cells and other cell types following exposure to a variety of stressful stimuli (15, 16). Mild stresses induce heat shock proteins and allow mammalian cells to adapt to gradual changes in their environment, which can protect heart and brain against ischemia, and lungs and liver against sepsis (17-20). Specifically, exposure of cardiac cells to a mild thermal or ischemic stress, sufficient to induce HSP expression, is protective against subsequent exposure to more severe ischemic stress

(13, 21, 22). The involvement of heat shock proteins, such as hsp70, hsp90, hsp 27 and αB-crystallin, in myocardial protection has been shown in cultured cells of cardiac origin (21, 23-26) as well as *in vivo* using transgenic mice (27-30). A decreased temperature of hearts also reduces infarct size in various animals (31-34), which might be associated with the cold stress response.

Tetracycline, an antibiotic, binds to ribosomes and inhibits translation in *E. coli*, which triggers the induction of the cold stress response (35). Recently, the induction of cold stress response by the antibiotic has been found to enhance the heterologous expression of enzymatically active proteins in *E. coli* (36-41). In mammals, tetracycline specifically inhibits mitochondrial translation (42), which led us to postulate that the inhibition of mitochondrial protein synthesis by tetracycline might induce unknown cellular stress response in mammals as does in *E. coli*. In addition, doxycycline and minocycline, long lasting tetracycline derivatives, have been known to exert various non-antibacterial and beneficial effects (43-45). Therefore, we investigated the ability of tetracycline to protect the heart against ischemic injury and a putative association of stress response induced by tetracycline with myocardial protection.

3. MATERIALS AND METHODS

3.1. Hemodynamics and segment shortening in dogs

The coronary artery ligation using adult mongrel dogs weighing 5,5–22 kg was carried out with monitoring hemodynamic parameters and segment shortening as described previously (46). Myocardial segment shortening was measured by a pair of ultrasonic crystals implanted into the subendocardium. The two crystals of each pair were separated by approximately 1 cm. The crystal-transmitted sound pulse was monitored with an oscilloscope. Diastolic segment length (DL) was determined at the beginning of the rising phase of positive first derivative of left ventricular pressure LVdP/dT (onset of isovolumic contraction), and systolic segment length (SL) was determined at the peak negative LVdP/dT (47). The segment shortening was calculated by the equation of segment shortening = (DL-SL)/DL. The values of segment shortening were normalized to the respective pre-injection values and expressed as % changes.

Saline or tetracycline (4 mg/kg in saline) was intravenously injected in a volume of 0.5 mL/kg, 30 min prior to the ligation. The ligature around the coronary artery was tied for 20 min and released for reperfusion. Measurements of hemodynamic parameters were continued for further 60 min after releasing the ligature.

3.2. Coronary artery ligation in mice

Tetracycline (4 mg/kg) was intraperitoneally injected 0.5 or 1.5 hour prior to the ligation. Male mice (C57BL/6) weighing 25-30 g were anesthetized with ketamine/xylazine (100 mg/kg/10 mg/kg). When anterior coronary artery was ligated, ends of the suture were passed around a polyethylene tube to form a snare. Electrocardiogram (ECG) was monitored throughout the experimental protocol.

Mice were subjected to regional myocardial ischemia by tightening the snare. Ischemia was confirmed by cyanosis, regional akinesis, and ECG changes. At the end of the ischemic period (20 min), the snare was released by removing the inserted tube and reperfusion was confirmed by hyperemia. The snare was kept on the heart for retying later to delineate the risk zone by the infusion of Phthalo Blue dye.

3.3. Determination of the infarct size in murine hearts

The quantitative analyses of infarct size were performed by triphenyltetrazolium (TTC) and Phthalo Blue staining as described by Guo et al. (48) with modifications described below. After 20 min ischemia followed by 15 min reperfusion, 1% TTC in phosphate buffered saline (PBS) was infused from abdominal aorta (1 mL/min) for 5 min. Immediately after the TTC staining, the snare left on the heart was retied to reproduce the field of occluded artery (ischemic area) and 4% Phthalo Blue (suspension of insoluble blue pigments, Heucotech, Fairless, PA) in PBS was infused to visualize the non-ischemic area (stained blue by Phthalo Blue). The heart was excised, fixed by 4% paraformaldehyde and sliced into 0.4 mm thick sections by Vibratome (Oxford, Wilmington, NC). The color images of sections were analyzed by NIH Image. The infarct area and risk zone of sections from a heart were integrated and the ratio of infarct area and risk zone in volume was obtained.

3.4. Preparation of tissue samples for electron microscopic analysis

After 20 min occlusion (n=2 for each group), Bokuju (Japanese type of India ink) was infused through aorta to visualize the ischemic area. Tissues at the center of ischemic and non-ischemic area were immediately dissected in PBS containing 1% glutaraldehyde and 2% paraformaldehyde and processed for the electron microscopic analysis (Philips CM-12 electron microscope).

3.5. Identification of proteins induced by cold- and tetracycline- treatments in HeLa cells

HeLa cells cultured as described (49) were incubated on ice for 15 min and recovered at 37 ^{o}C in CO_2-incubator for 3 hours. HeLa cells were also treated with tetracycline (1 μg/mL medium) for 3 hours at 37 ^{o}C in CO_2-incubator. Cells were homogenized in the buffer containing 10 mmol/L Hepes (pH 7.9), 1.5 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 0.5 mmol/L EDTA, 0.1 mmol/L PMSF, and centrifuged at 20,000g x 10 min. The supernatants were analyzed by two-dimensional gel electrophoresis as manufacturer's instruction (the Protein IEF system, Bio-Rad) followed by MALDI-TOF mass spectrometry (50).

RNA was extracted from HeLa cells exposed to cold (4 ^{o}C x 15 min followed by 2 hours recovery) and tetracycline (1 μg/mL x 2 hour) utilizing an RNA preparation kit (RNAeasy, Qiagen). The microarray analysis was carried out as described (51). RNA (50 μg/reaction) was labeled with Cy3- or Cy5-labeled dUTP using oligo-dT and reverse transcriptase (Superscript II RT,

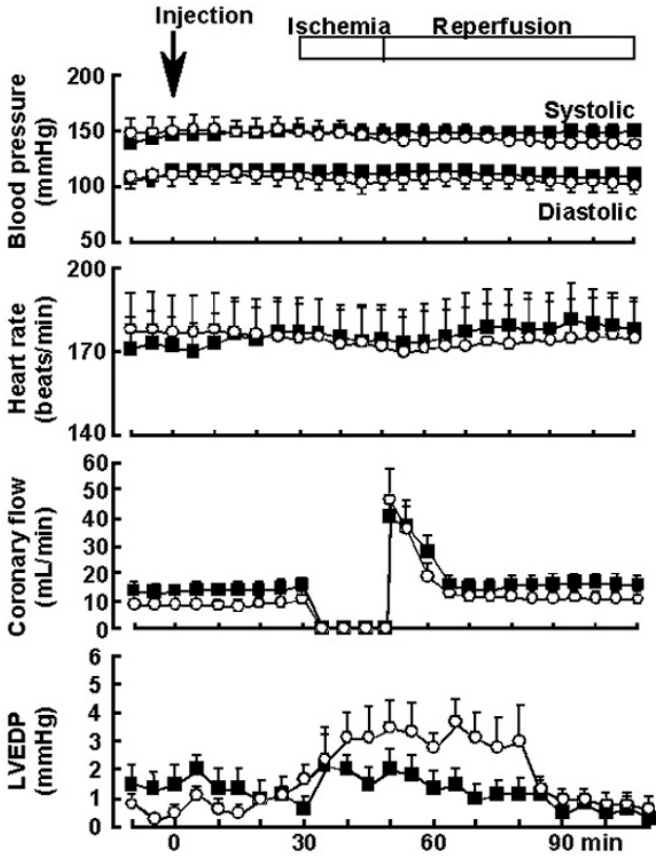

Figure 1. Hemodynamic parameters during ischemia and reperfusion. Blood pressure, heart rate, coronary flow, and left ventricular end diastolic pressure were monitored throughout the experimental procedure for both control (open circle, n=6) and tetracycline-treated dogs (closed square, n=6).

LifeTechnologies). When RNA profiles of control and tetracycline-treated cells were compared, one array (containing 5,000 individual cDNA clones) was hybridized with Cy3-labeled probe from control RNA and Cy5-labeled probe from tetracycline-treated RNA, and the other array was hybridized with the opposite combination of labeling. After hybridization and washing, slides were scanned using Scan Array 3000 (GSI Lumonics) and analyzed using an image processing software TIGR-Spotfinder. Using a post-nomalization cutoff of two-fold up- or down-regulation, four clones (1-4 in Figure 6) were identified and their nucleotide sequences were determined by automated sequencing.

3.6. Immunohistochemical detection of stress response proteins in murine hearts

Anti-HSP27 and HSP70 were purchased from Santa Cruz (Santa Cruz, CA), anti-TGase II was from Lab Vision (Fremont, CA), anti-profilin 1 from Cytoskeleton (Denver, CO) and anti-cyclophilin A from Calbiochem (San Diego, CA). After 24 hour permanent ligation, murine hearts were infused by Bokuju for the determination of the risk zone and non-risk zone, fixed, cut into two pieces along the long axis and embedded in paraffin. Immunohistochemical staining was carried out as described (52). Representative data obtained from three mice are shown.

3.7. Statistical analysis

Data are expressed as mean±S.E.M. Statistical significance was calculated using Student's t-test for comparison of two groups, and analysis of variance followed by Dunnett's post hoc procedures as appropriate. A value of $P<0.05$ was considered statistically significant.

4. RESULTS

4.1. Improvement of functional recovery by tetracycline during reperfusion

The coronary artery ligation, an experimental model of myocardial ischemia, was performed using dogs. A pair of ultrasonic crystals was implanted in the ischemic region to monitor the segment length for analysis of segment shortening caused by ischemia, which provides information on the functional recovery of hearts from ischemic stress during reperfusion. Saline (Cont) or tetracycline (Tet), 4 mg/kg body weight, was injected 30 min prior to the occlusion. After 20-min ischemia, the hearts were reperfused for 1 hour. Although the patterns of blood pressure, heart rates, and coronary flow were similar between control and tetracycline-treated groups, the left ventricular end-diastolic pressure (LVEDP) was more stable in the tetracycline-treated group (Tet) (Figure 1).

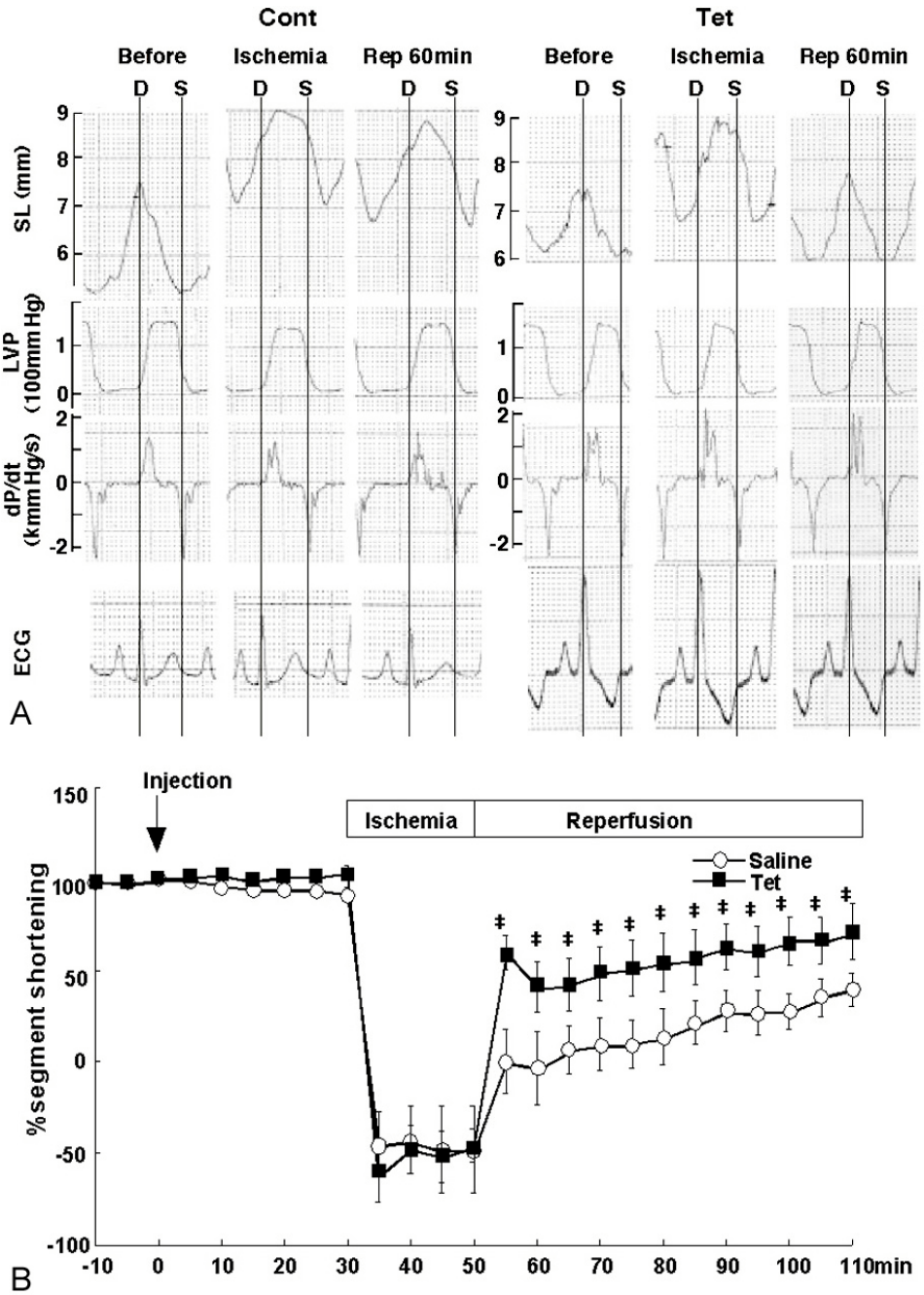

Figure 2. Effect of tetracycline on the segment length. A, representative charts of segment length (SL), left ventricular pressure (LVP), the first derivative of left ventricular pressure (dP/dt), and electrocardiogram of control dogs (Cont) and tetracycline-treated dogs (Tet) were shown for before ischemia (Before), during ischemia (Ischemia), and after 60 min reperfusion (Rep 60min). Vertical lines marked D and S indicated the positions of diastolic and systolic segment length, respectively. B, percent segment shortening was analyzed from the segment length in control (open circle) and tetracycline-treated dogs (closed square). ‡ Significantly different from control group (P<0.05).

The wave patterns of segment length were shifted by the occlusion in both controls and Tet (Figure 2A). After 60 min reperfusion, the shift in the segment length recovered to the normal position in Tet but not in control. The end-diastolic and end-systolic segment lengths identified as described in "Methods" were analyzed throughout the experiments for comparison of segment shortenings between controls and Tet. The percentage of segment shortening (%SS) fell below 0% during ischemia similarly in both control and Tet (Figure 2B). After reperfusion, however, Tet clearly showed the improvement in the recovery of the %SS compared with control although the

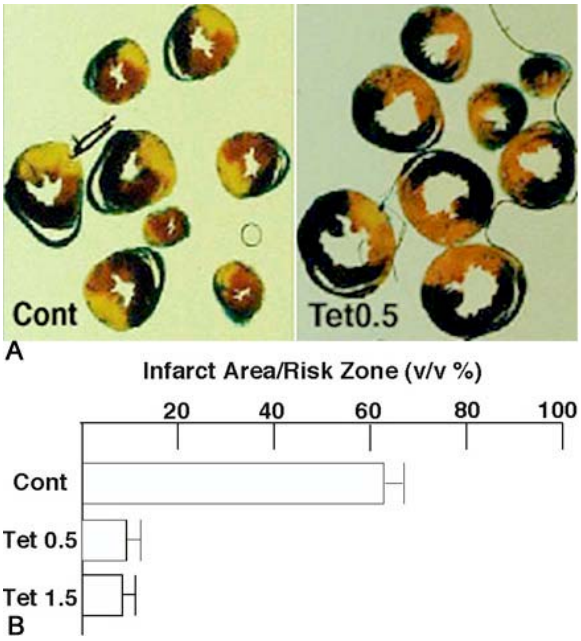

Figure 3. Quantitative analysis of infarct size in murine hearts. **a**, representative slices stained by TTC and Phthalo Blue are shown for control (Cont) and tetracycline-treated mice (Tet0.5). B, Protective effect by tetracycline was analyzed by the ratio in volume of infarct area (area of pale tan in A) and the risk zone (area stained red in A). Tetracycline was administered 0.5 (Tet0.5) or 1.5 hour (Tet1.5) prior to the ligation. The values were the mean of 9 for control, 9 for Tet0.5, and 6 mice for Tet1.5. The values of Tet0.5 and Tet1.5 were significantly different from control (P<0.01).

complete recovery was not obtained in 1 hour reperfusion. This improvement of functional recovery from stunned myocardium suggests a cardioprotection by tetracycline from ischemic injury.

4.2. Protection of cardiac myocytes by tetracycline from ischemic injury

Because stunned myocardium is not necessarily accompanied by myocardial infarction, the effects of tetracycline on infarct caused by myocardial ischemia were investigated utilizing a mouse model. In similar ischemia and reperfusion experiments, the effect of tetracycline was analyzed by the combination of triphenyl-tetrazolium (TTC) and Phthalo Blue staining. TTC is converted to insoluble red pigments (a formazan compound) by a reducing reaction in active mitochondria. At the end of reperfusion, 1% TTC solution infused from the abdominal aorta stained surviving cardiac tissue deep red and left the infarct tissues pale tan. After TTC staining, the suture left on the heart during reperfusion was retied to reproduce the field of occluded artery (the risk zone). Then Phthalo Blue, a colloidal insoluble dye, was infused to delineate the risk zone. Thus, the non-risk zone stained deep red by TTC was also stained blue by Phthalo Blue, yielding the black area in Figure 3A. The surviving tissues within the risk zone were stained deep red by TTC alone and the infarct area within the risk zone was left unstained. By this methodology, the major portion of the risk zone was infarcted in hearts from control mice (Cont) (Figure 3A). However, hearts from Tet showed infarct tissues only close to the suture or, in some cases, showed small patchy infarct areas. Utilizing the stained images, the ratio in volume of the infarct area and the risk zone was analyzed by integrating the areas of slices from each heart (Figure 3B). The administrations of 4 mg/kg tetracycline at 0.5 and 1.5 hour prior to the ischemia similarly reduced the infarct size to less than 10% of the risk zone in contrast to 63% in control (Figure 3B). When 4 mg/kg tetracycline is injected in mice, tetracycline level in serum ranges between 1 to 2 μg/mL 30 min after injection and decreases with a half-life ($t_{1/2}$) of approximately 1 hour (53). Therefore, its serum concentration will be less than 1 μg/mL in 1.5 hour after injection, suggesting that tetracycline may be effective for cardioprotection at a very low concentration, or that the cellular response may last for a long duration once it is triggered by tetracycline.

The conversion of TTC to insoluble red pigments reflects mitochondrial function (54). Therefore, mitochondria in the infarct area determined by TTC staining should be damaged during the 20 min occlusion in control but not in Tet. To eliminate the possibility that tetracycline might enhance the TTC conversion to the red pigments via an unknown mechanism, we examined effects of 20 min coronary artery ligation on cellular ultrastructure by electron microscopy. In control animals, heart tissues at the center of the risk zone showed severe irreversible damage in most mitochondria (mitochondrial swelling, disruption of cristae, and amorphous matrix densities) (Figure 4A), whereas tissues from the ischemic area in tetracycline-treated animals showed little damage of mitochondria (Figure 4B), and looked similar to the tissues from the non-risk zone of control and Tet (not shown). These cellular ultrastructural results are consistent with those from TTC staining, clearly indicating that tetracycline protects heart from ischemic injury. The ultrastructural results also confirm reliability of the quantitative analysis of infarct size determined by TTC staining in the present system.

4.3. Proteins induced by tetracycline and cold stress in HeLa were also induced by ischemic stress in murine hearts

Tetracycline mimics cold stress triggered by translational inhibition and induces a series of cold stress response proteins in *E. coli* (35), suggesting that a mild inhibition of mitochondrial translation by tetracycline may cause cellular stress and induce a series of stress response proteins in mammalian system. Therefore, we investigated the profiles of protein extracts from HeLa cells treated by tetracycline by two-dimensional gel electrophoresis. As shown in Figure 5, spots A and B were clearly induced by both cold- and tetracycline-treatments compared with untreated control. These proteins were digested by trypsin and the resulting peptides were analyzed by MALDI (Matrix Assisted Laser Desorption-Ionization) mass spectrometry analysis. As shown in Table 1, peptide mass from spots A and B matched with human cyclophilin A and profilin 1, respectively. We also analyzed mRNA from

Table 1. Identification of proteins on a 2-D gel by MALDI-TOF mass spectrometry. Tryptic peptides from spots A and B indicated in Figure 5 were analyzed by mass spectrometry. Measured peptide mass and corresponding computed mass and peptide sequences were shown. M-ox indicates peptides containing oxidized methionine.

Spot A	**Human cyclophilin A**	
Measured Mass	Computed Mass	Peptide Sequence
762.351	762,323	GSCFHR
1054.583	1054.533	VSFELFADK
1153.626	1153.626	FEDENFILK
1246.697	1246.622	KITIADCGQLE
1277.646	1277.574	EGMNIVEAMER
1597.748	1597.737	IIPGFMCQGGDFTR
1613.716	1613.732	IIPGFMCQGGDFTR (M-ox)
1945.078	1944.994	VNPTVFFDIAVDGEPLGR
2806.364	2806.315	HTGPGILSMANAGPNTNGSQF
Spot B	**Human Profilin 1**	
Measuered Mass	Computed Mass	Peptide Sequence
1165.487	1165.500	CYEMASHLR
1181.472	1181.495	CYEMASHLR (M-ox)
1212.582	1212.613	DSPSVWAAVPGK
1378.735	1378.708	STGGAPTFNVTVTK
1469.780	1469.751	SSFYVNGLTLGGQK
1624.713	1624.740	DSLLQDGEFSMDLR
1640.661	1640.734	DSLLQDGEFSMDLR (M-ox)
1642.807	1642.929	TFVNITPAEVGVLVGK

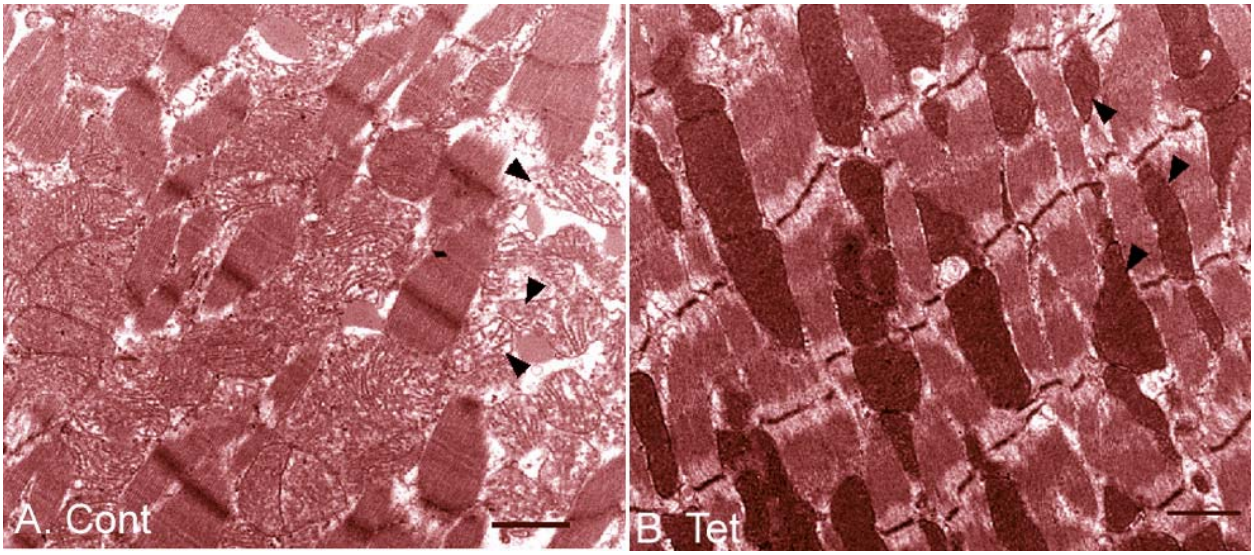

Figure 4. Electron micrographs obtained from ischemic heart tissues after 20 min occlusion. A, electron micrograph from ischemic heart tissues of control mice. Mitochondria show swelling (matrix clearing) and severe architectural disruption of cristae as well as amorphous matrix densities. Intracellular edema is also seen. (Direct magnification = 15,000x; bar = 500 nm). B, electron micrograph from ischemic tissues of tetracycline-treated mice. The mitochondria appear normal and sacromers are intact. (Direct magnification = 11,500x; bar = 500 nm).

HeLa cells exposed to tetracycline using cDNA microarray analysis. We found that tetracycline as well as cold stress induced tissue transglutaminase II (Figure 6). These results suggest that tetracycline may induce cold stress-like response in mammalian cells.

The mild stress by heat-pretreatment of tissues or even whole animals induces a series of stress response proteins, such as HSP70 and HSP27, and protects myocardium from ischemic injury (13, 26, 55). These stress response proteins are also induced by ischemic stress (22, 56). Therefore, if the stress response caused by tetracycline via the inhibition in mitochondrial translation involves targets of cardioprotection, the tetracycline-inducible genes may also be induced by ischemia. Using a murine model of ischemia, we carried out the coronary artery ligation for 24 hours and examined the expression of the profilin 1, cyclophilin A, and transglutaminase II as well as HSP70 and HSP27 by immunohistochemical analysis. In parallel with HSP70 and HSP27, the expression levels of all three

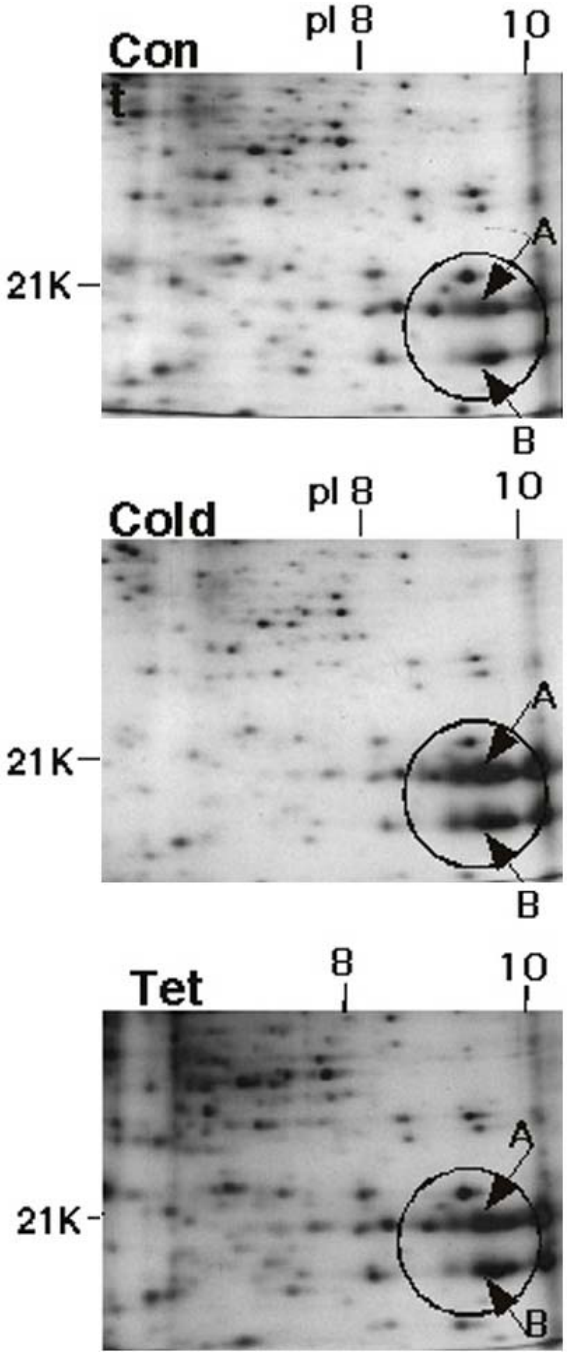

Figure 5. Two-dimensional gel electrophoresis of proteins induced by both cold-stress and tetracycline**.** HeLa extracts (0.5 mg) from control, cold-treated, and tetracycline-treated cells were applied on isoelectric focusing followed by SDS-PAGE electrophoresis. A and B indicate the proteins induced by both cold- and tetracycline-treatment.

proteins were significantly elevated in the risk zone compared with the non-risk zone (Figure 7). The level of all three proteins that were induced by tetracycline in HeLa were increased in murine hearts by ischemic stress, suggesting that the three proteins may be stress response proteins. Although these proteins may not be directly involved in the early phase of cardioprotection, a stress response induced by tetracycline may very well be associated with the cardioprotection from ischemic damage.

5. DISCUSSION

Tetracycline derivatives have been reported to exert non-antimicrobial effects at high dosages (40-180mg/kg), such as anti-inflammatory properties (43, 57-60).

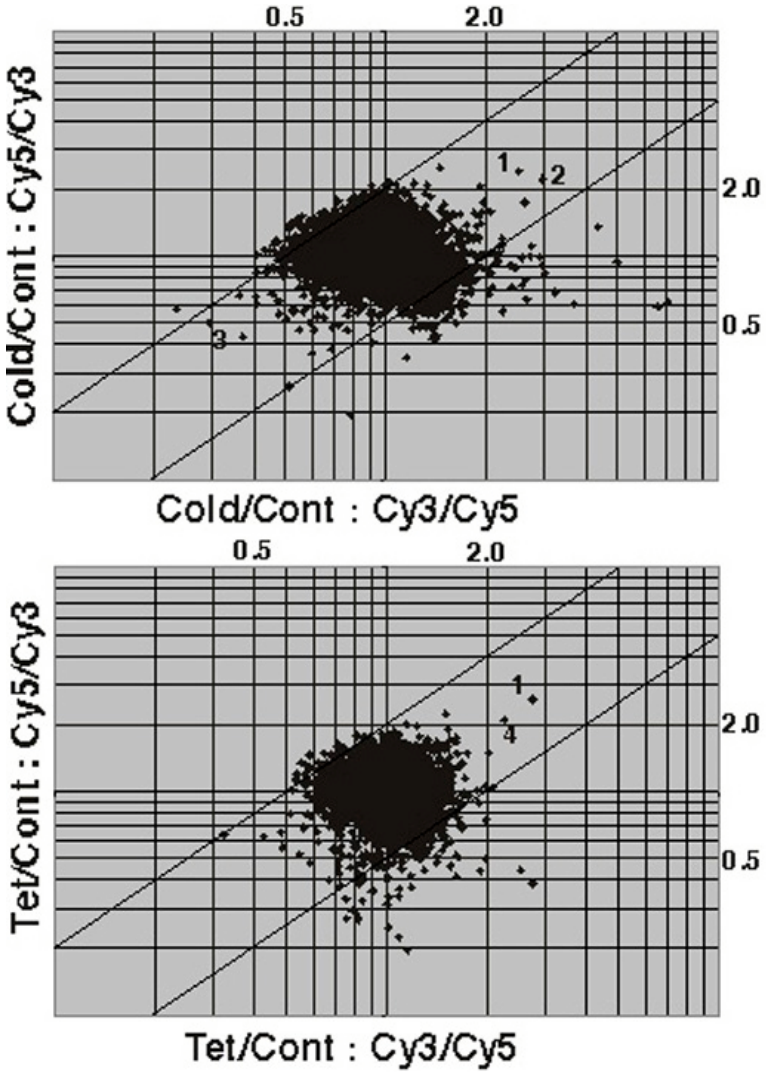

Figure 6. Profiles of mRNA from HeLa cells treated with cold stress and tetracycline.The cDNA microarray analysis was carried out as described in "Methods and Materials". The ratios of fluorescence intensities were normalized as described.(51) 1, tissue transglutaminase II was induced 2.5±0.1 fold by cold stress and 2,7±0.1 by tetracycline; 2, trombospondine 1 was 2.6±0.5 fold by cold; 3, caspase 9 was down-regulated 0.41±0.04 fold by cold stress; 4, insulin-induced gene 1 was induced 2.2±0.1 fold by tetracycline.

Minocycline also mediates neuroprotection in experimental models of cerebral ischemia (44, 61) and amyotrophic lateral sclerosis (45). Although minocycline inhibits the upregulation and activation of cell death mediators such as caspases, cyclooxygenase-2, interleukin-1□-converting enzyme, inducible nitric oxide synthase, and cytochrome c release, the upstream mechanisms by which minocycline triggers the interference in upregulation and activation of the cell death mediators have not been well investigated.

In the present study, we have demonstrated a remarkable cardioprotection by tetracycline. After the administration of tetracycline (4mg/kg), serum tetracycline reaches 4-5 μg/mL in 15 min, decreases to 1-2 μg/mL in 30 min, and declines with an approximate $t_{1/2}$ of 1 hour in mice (53) and 3 hours in dogs (62). Tetracyclines in the range of 5-10 μg/mL serum are required to inhibit mitochondrial translation for arresting tumor proliferation (63) or to inhibit other enzyme activities, e.g., metalloproteinases (64). Thus, the serum concentration of tetracycline during occlusion and reperfusion are too low to efficiently inhibit mitochondrial protein synthesis or enzyme activities. Similar to the neuroprotection by minocycline, therefore, molecular mechanisms underlying the cardioprotection by tetracycline remain to be investigated. The concentration of 1-2 μg/mL tetracycline is not sufficient for complete inhibition of bacterial translation but is high enough for partial inhibition of protein synthesis to trigger cold stress response in *E. coli* (35, 38). A similar concentration of tetracycline in the culture media also induced the proteins, profilin 1, cyclophilin A, and transglutaminase II, in HeLa cells as cold stress did (Figure 5 and 6). These proteins

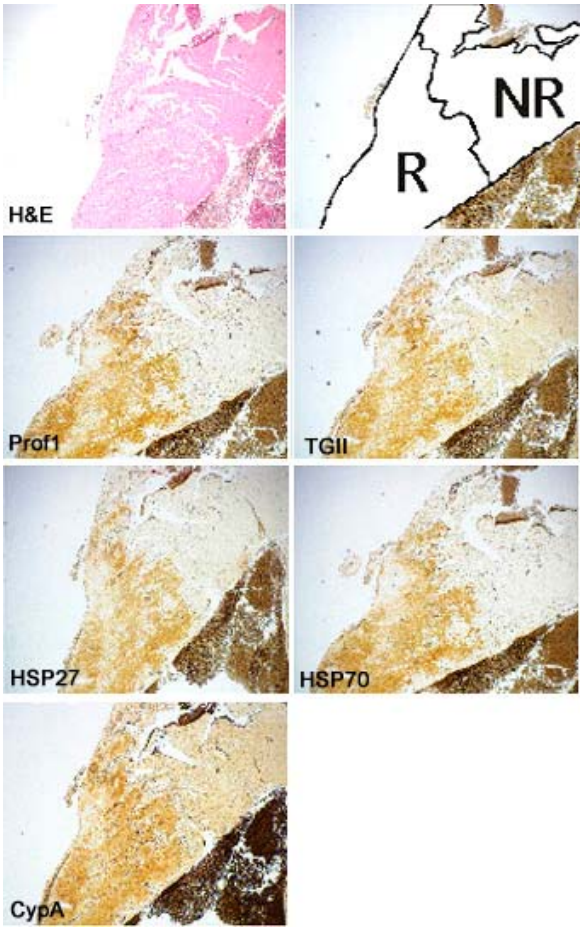

Figure 7. Induction of profilin 1, cyclophilin A and tissue transglutaminase by ischemic stress.Consecutive slices from a heart subjected to 24 hour ischemia without reperfusion were analyzed by immunohistochemical staining. R and NR in right upper panel indicate risk zone and non-risk zone. H&E, hematoxylin and eosin staining; Prof1, profilin 1; TGII, tissue transglutaminase; CypA, cyclophilin A; HSP27, heat shock protein HSP27; HSP70, heat shock protein HSP70.

were also induced during ischemic stress (Figure 7). It is important to note that a low concentration of tetracycline appears to induce a series of proteins that are induced by cold stress and ischemic stress. Because stress response is the fundamental mechanism observed from bacteria to animal cells, a partial inhibition of mitochondrial translation by tetracycline may also trigger cellular stress response in mammalian cells as in *E. coli*. Therefore, the tetracycline-mediated stress response triggered by translational inhibition in mitochondria may be associated with the cardioprotective effect against ischemic injury.

In heart, repeated 5-min coronary artery occlusions followed by 5-min reperfusions do not develop infarction but trigger cardioprotection (48) (known as ischemic preconditioning). The myocardial infarction caused by 30 min occlusion is not further developed at least after 4-hour reperfusion (48). When the activation and infiltration of inflammatory cells are involved, the infarction develops in several days after ischemia (61). These studies suggest that direct damage on cardiac myocytes may be the major cause of infarct in the regional myocardial ischemia. Therefore, tetracycline appears to directly protect cardiac myocytes from ischemic injury rather than to arrest activation of inflammatory cells. Thus, we hypothesize that a subclinical dosage of tetracycline may protect cardiac myocytes from ischemic injury by virtue of the capacity to induce stress response, probably cold stress-like response as in *E. coli*, via a partial inhibition of mitochondrial translation. This hypothesis of tetracycline-mediated stress response may provide a new therapeutic target for myocardial protection against ischemic injury.

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All authors equally contribute to this study.

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