Genistein Attenuates Postischemic Depressed Myocardial Function by Increasing Myofilament Ca^{2+} Sensitivity in Rat Myocardium[1]

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The present study investigated whether genistein, a broad-spectrum tyrosine kinase inhibitor, could increase the myofilament Ca^{2+} sensitivity and partially reverse postischemic depressed myocardial function. Left ventricular papillary muscles were isolated from adult Wistar rats and loaded with the Ca^{2+} indicator, aequorin. The use of fluorocarbon immersion with hypoxia simulated a model of ischemia. Myofilament responsiveness to Ca^{2+} was evaluated from force-$[Ca^{2+}]_i$ relationship recorded during tetani in papillary muscles. Protein levels of troponin I (TnI) were measured in postischemic papillary muscles with the Western blot technique. Isometric contraction was depressed during the period of ischemia and remained low after 60 min of reoxygenation without a corresponding significant change of peak $[Ca^{2+}]_i$ in the control group ($n = 7$). In contrast, the depression of isometric contraction was ameliorated during ischemia in muscle preparations in the presence of genistein (2 μM; $n = 8$), and postischemic depressed myocardial contractility partially recovered after a 60-min reperfusion. The myofilament Ca^{2+} responsiveness was significantly increased in papillary muscles in the presence of genistein. Protein levels of TnI were reduced in postischemic papillary muscles, whereas genistein partially restored decreased protein levels of TnI. Our results reveal that genistein produces an effective attenuation of postischemic depressed myocardial function and improves myofibrillar Ca^{2+} responsiveness in rat myocardium.

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Key words: genistein; intracellular Ca^{2+}; ischemia; troponin I

It has been demonstrated that impaired intracellular Ca^{2+} ($[Ca^{2+}]_i$) modulation is one of the primary factors in the pathogenesis of postischemic myocardial stunning (1–3). Analysis of experimental models has localized the impairment of the contractile machinery to a reduction of myofilament Ca^{2+} responsiveness (1, 2, 4). A loss of TnI from the cell, the altered structure of TnI, and the altered function and interactions of TnI are the possible events in ischemia-reperfusion injury (5). Kusuoka and Marban (3) suggested that stunning is associated with a reversible breakdown and replacement of damaged myofilament proteins. Additionally, McDonough *et al.* (5) reported that altered thin-filament regulation with TnI degradation resulted in myocardial stunning and more severe episodes of ischemia with and without reperfusion. Huang *et al.* (6) showed diastolic dysfunction and reduced myofilament responsiveness to Ca^{2+} in TnI gene-ablated mouse hearts. We hypothesize that myocardial stunning might also be associated with phosphorylation of TnI in addition to proteolysis and degradation of TnI during the ischemia-reoxygenation cycle.

Activation of protein kinase C (PKC) and protein kinase A (PKA) by increased catecholamine levels during the ischemia-reperfusion cycle, via stimulation of the α-adrenoreceptor and β-adrenoreceptor, respectively, leads to activation of the Raf-1 kinase/extracellular signal-regulated protein kinase (ERK) cascade (7, 8). This activation subsequently results in phosphorylation of TnI, decreased Ca^{2+} sensitivity, or decreased activity of actomyosin MgATPase and a decreased rate or affinity of Ca^{2+} binding to troponin C (TnC) (9, 10). It has been accepted that tyrosine kinases are responsible for activation of ERKs induced by isoproterenol in cardiac myocytes (11). Stimulation of tyrosine kinase by activated ERKs may contribute to the postischemic ventricular dysfunction via phosphorylation of TnI. Activation of ERKs by isoproterenol was suppressed completely when cardiomyocytes were pretreated with the broad-spectrum tyrosine kinase inhibitor, genistein (11). The aim of this study was to investigate whether genistein

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may partially reverse postischemic ventricular dysfunction by increasing the myofilament Ca^{2+} sensitivity resulting from inhibiting the activation of tyrosine kinase.

Methods

The experiments were performed in male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 250–300 g. Animals were housed individually under climate-controlled conditions with a 12:12-hr light:dark cycle and free access to a standard rat chow and tap water *ad libitum*. The study was conducted under the guidelines for *The Care and Use of Laboratory Animals* published by the U.S National Institutes of Health (NIH Publication 85-23, revised 1996), and the experimental protocol was approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center.

Isolated Muscle Performance. The rats were sacrificed under deep pentobarbital anesthesia. Each heart was rapidly excised and placed in a dissecting chamber containing a modified Krebs-Henseleit solution of the following composition (in millimoles): NaCl 120, KCl 5.9, dextrose 5.5, $NaHCO_3$ 25, NaH_2PO_4 1.2, $MgCl_2$ 1.2, and $CaCl_2$ 1.0, pH 7.4, bubbled with carbogen (a mixture of 95% O_2 and 5% CO_2) at room temperature. A left ventricular posterior papillary muscle was carefully dissected and fixed to a muscle holder with a spring clip. The tendinous end of the muscle was vertically connected to a strain-gauge tension transducer (Model MBI 341; Sensotec, Columbus, OH) with a silk thread. The muscle was then mounted in a 50-ml tissue bath containing modified Krebs-Henseleit solution maintained at 30°C and continuously bubbled with carbogen. The isometric contraction of the papillary muscle was elicited by a punctate platinum electrode with square-wave pulses of 5 ms duration at 0.33 Hz. The voltage was set to 10% above threshold level. After a 30-min equilibration period, the muscle was carefully stretched to the length at which maximal tension developed. Developed tension (tension produced by the stimulated muscle) was recorded from each muscle at this maximal length. Subsequently, the loading procedure for aequorin was performed (see below).

Aequorin Light Signal Measurement. Aequorin (Dr. John Blinks, Friday Harbor Laboratory, San Juan Island, WA) was loaded by the macroinjection technique (12). Briefly, the muscle preparation was raised from the organ bath and 1–2 μl of aequorin solution (2 mg/ml) was injected under the epimysium at the base of the muscle with a short-shank low-resistance glass micropipette. After an equilibrium period of 90–120 min, the stimulation was restarted at 0.33 Hz. The aequorin light signal was detected with a photomultiplier tube (PM28B; Thorn EMI Electron Tubes, Rockaway, NJ) and converted into a voltage signal. The analog signals from the isometric force transducer and electronic photometer were recorded with a chart-strip recorder (Model 56-1X 40-006158; Gould Instrument Systems, Cleveland, OH). To improve the signal-to-noise ratio, 64–128 steady-state light signals and isometric twitches were averaged with a digital oscilloscope (Model 4094; Nicolet Instrument, Madison, WI). The free intracellular concentration of calcium ($[Ca^{2+}]_i$) was estimated by normalizing the recorded light signal during the isometric twitch with the maximal amount of light emitted after lysis of the muscle membranes at the end of the experiment with a 5% solution of the detergent Triton X-100 in phosphate-free physiological salt solution containing 50 m*M* Ca^{2+}. The normalized light signal was then converted to $[Ca^{2+}]_i$ using an *in vitro* calibration curve as previously reported (2, 12).

Simulated-Ischemia Model with Fluorocarbon. Steady-state conditions were observed for at least 30 min. After measuring the baseline parameters in physiological salt solution (PSS) with oxygenation (95% O_2 and 5% CO_2), isolated papillary muscles were exposed to oxygenated fluorocarbon (Fluorinert [FC-47]; 3M Co., St. Paul, MN) and were equilibrated for a 30-min period. After this, ischemia was induced by exposing muscle preparations to a 20-min period of hypoxia (95% N_2 and 5% CO_2) with fluorocarbon immersion. Subsequently, reoxygenation was repeated in rat muscles with fluorocarbon for 60 min. Failure of the developed tension to return to preischemic condition was taken as evidence of postischemic myocardial stunning.

Experimental Protocol. Steady-state conditions were observed for at least 30 min after the intracellular aequorin light signal had stabilized. After measurement of all parameter baseline values and the values at fluorocarbon immersion, effective doses of genistein (2 μ*M*) were added into the organ bath in the genistein-pretreated group (n = 8), whereas no drug was added in the control group (n = 7). Steady-state measurements of contractility and aequorin light signals were obtained 30 min after the addition of genistein. Subsequently, a simulated-ischemia model with fluorocarbon immersion was performed in all muscle preparations as mentioned above.

Steady-State Force-$[Ca^{2+}]_i$ Relations. Myofilament responsiveness to Ca^{2+} was evaluated by force-$[Ca^{2+}]_i$ relationships recorded during tetanus (13). In the mammalian cardiac muscle, complete fusion of individual tetani can be accomplished by prior inhibition of sarcoplasmic reticulum function with ryanodine (14). After reoxygenation for 60 min in the fluorocarbon immersion, 0.2 μ*M* ryanodine was added into the bath, which caused a reduction in isometric force and $[Ca^{2+}]_i$. Steady state was reached approximately 20 min after adding ryanodine in the condition of fluorocarbon immersion. Muscle tetanus was induced by stimulating the intact papillary muscle at 15 Hz for 5 s using stimulus pulses 50 ms in duration with field electrodes. Tetanus was elicited at varying extracellular Ca^{2+} concentrations of 1.0, 2.0, 4.0, 8.0, and 12.0 m*M*. Our preliminary experiments suggested that there was no further increase in tetanic force at higher extracellular Ca^{2+} levels above 8.0 m*M*. Phosphate was removed from the bath medium during these determinations to avoid Ca^{2+} precipitation. The muscle developed tension versus $[Ca^{2+}]_i$ curves were fitted to the Hill equation:

$$T = T_{max} \frac{[Ca^{2+}]_i^n}{[Ca^{2+}]_{i50}^n + [Ca^{2+}]_i^n}$$

where T is developed tension of the papillary muscle, T_{max} is maximal developed tension, n is Hill coefficient, and $[Ca^{2+}]_{i50}$ is $[Ca^{2+}]_i$ required for 50% activation.

Immunoblotting. Total proteins (five for each) extracted from nonischemic and postischemic papillary muscles with or without pretreatment of genistein were analyzed for expression of TnI by immunoblotting. Briefly, muscle preparations were lysed in a lysis buffer (137 m*M* NaCl, 20 m*M* Tris-HCl, pH 7.4, 20 m*M* NaF, 1 m*M* Na-pyrophosphate, 1 m*M* vandate, 10% glycerol, 4 μg/ml leupeptin, 4 μg/ml pepstain, and 1 mM phenylmethylsulfonyl fluoride). The protein concentrations were determined by the Biaford method (Bio-Rad, Hercules, CA). Cell lyses containing 50 μg/lane proteins were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and were transferred onto a polyvinylidene difluoride membrane (Micron Separations, Westboro, MA). After transfer, the membrane was blocked in 5% fat-free milk in Tris-buffered saline/Tween 20, and was then probed by primary monoclonal antibodies to cardiac TnI (IgG2b, 41-SASRKLQLK-49; Research Diagnostics, Flanders, NJ). Bound primary antibody was detected using horseradish peroxidase-conjugated second antibody and was developed by an enhanced chemiluminescence Western blot kit (Amersham, Buckinghamshire, UK). All experiments were carried out three times, and cyclophilin A was employed as an internal control.

Statistical Analysis. Data are expressed as mean ± SD. Baseline results were analyzed using Student's *t* test for paired data. Intergroup comparisons were made with Student-Newman-Keuls multiple comparison tests. For ischemia-reoxygenation cycle studies, two-way analysis of variance (ANOVA) was used for intergroup comparisons. Significance was defined at the level of $P < 0.05$.

Results

Effect of Genistein on Mechanical Function and Intracellular Ca^{2+}. Measurements of mechanical parameters and intracellular Ca^{2+} signals in rat papillary muscles are presented in Figure 1. Baseline developed tension was almost identical in all animals. After baseline measurement, papillary muscles were exposed to a 30-min period of fluorocarbon immersion bubbled with oxygen. No significant difference in isometric contractility or intracellular Ca^{2+} was found in either group with fluorocarbon (Fig. 1). Genistein (2 μ*M*) was then added into the bath chamber and did not produce a significant change in either developed tension or $[Ca^{2+}]_i$.

Ischemia was produced for 20 min by immersing the muscle preparations into fluorocarbon bubbled with a mixture of 95% N_2 and 5% CO_2. The isometric force decreased without a parallel reduction of peak $[Ca^{2+}]_i$ (Figs. 1 and 2) in either control or genistein pretreated groups. After 60 min of reoxygenation, postischemic depression of myocardial function was found in all muscle preparations. Pretreatment with genistein resulted in partial reversion of postischemic depressed myocardial function in rat papillary muscles. When switching back to physiological salt solution, the isometric contractility and $[Ca^{2+}]_i$ availability promptly recovered to near baseline values (data not shown) and these phenomena are consistent with our previous findings (16, 17).

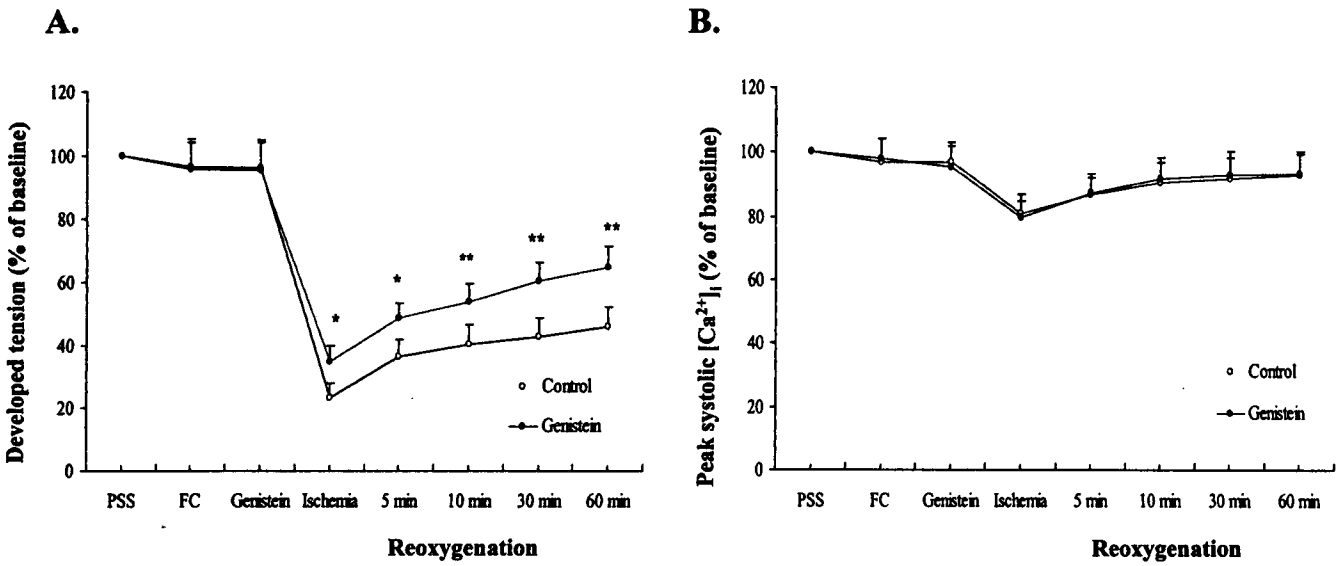

Figure 1. Developed tension (A, percentage of baseline values) and peak systolic $[Ca^{2+}]_i$ concentration (B, percentage of baseline values) in response to fluorocarbon simulated-ischemia model in papillary muscles isolated from adult rats. Control, rat papillary muscles without genistein ($n = 7$); Genistein, rat papillary muscles in the presence of genistein (2 μ*M*; $n = 8$). PSS, values in the physiological salt solution with oxygenation; FC, values in the fluorocarbon immersion with oxygenation; Genistein, values in the presence of genistein (2 μ*M*). $^*P < 0.05$; $^{**}P < 0.01$ Genistein vs. Control.

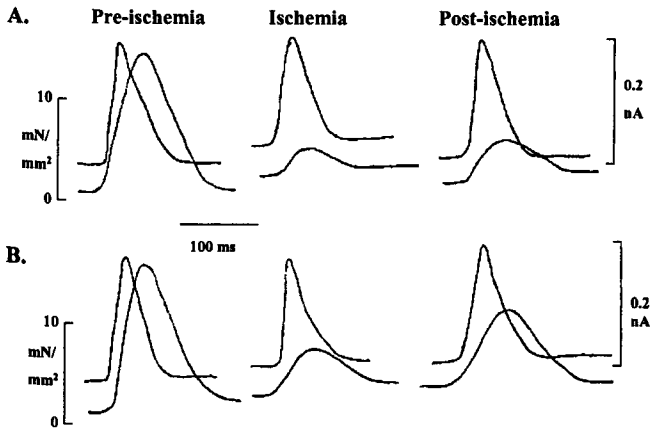

Figure 2. Aequorin light signal and isometric contraction from representative rat papillary muscles without genistein (A) and in the presence of genistein (B) during fluorocarbon simulated ischemia-reoxygenation cycle. Upper trace, aequorin light signal; Lower trace, isometric contraction.

Effect of Genistein on Myofilament Responsiveness to Ca^{2+}. Changes in the force-peak $[Ca^{2+}]_i$ do not accurately reflect myofilament responsiveness to calcium (13, 14). We used force-$[Ca^{2+}]_i$ relations recorded during the steady-state condition of tetanus as an index of the sensitivity of the myofilaments to Ca^{2+}. Figure 3 illustrates the effect of increasing $[Ca^{2+}]_o$ on tetanic force and Ca^{2+} transients in muscle preparations pretreated without genistein (Fig. 3A) or with genistein (Fig. 3B). Genistein pretreatment significantly increase sensitivity of the myofilament to Ca^{2+} compared with that in papillary muscles without genistein. Developed force and steady-state levels of $[Ca^{2+}]_i$ with tetanus as a graded increase of $[Ca^{2+}]_o$ are presented in Figure 4. Tetanic force was presented at 2.0, 4.0, and 8.0 m*M* of $[Ca^{2+}]_o$ due to no further increase in tetanic force at higher $[Ca^{2+}]_o$. The force-$[Ca^{2+}]_i$ curve of the control group was shifted to the right, whereas the force-peak $[Ca^{2+}]_i$ curve was shifted to the left in genistein-pretreated papillary muscles. Steady-state force-peak $[Ca^{2+}]_i$ curves for the three groups (Fig. 3) were fitted to the Hill equation. Maximum muscle developed tension was calculated as 12.6 ± 1.3 mN/mm^2 in the normal group, 9.8 ± 0.9 mN/mm^2 in the control group, and 14.7 ± 1.5 mN/mm^2 in the genistein-treated group. Half-maximal $[Ca^{2+}]_{50}$ was calculated as 0.59 ± 0.02 μ*M* in the normal group, 0.68 ± 0.04 μ*M* in the control group, and 0.54 ± 0.01 μ*M* in the

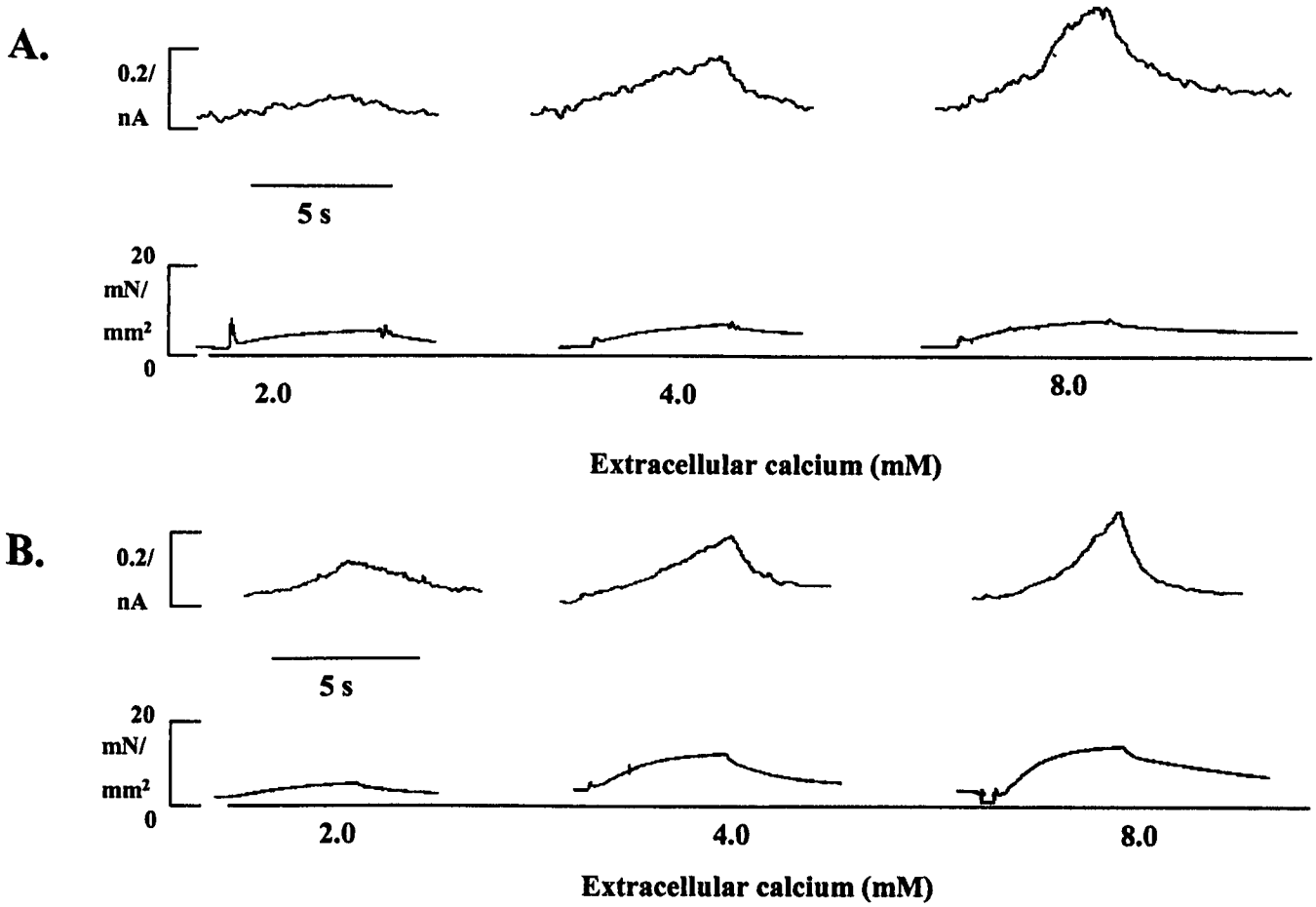

Figure 3. Representative examples of aequorin light signal and isometric contraction during tetanic stimulation from rat postischemic papillary muscle without genistein (A) and postischemic papillary muscle in the presence of genistein (2 μ*M*; B). Tetanus was elicited in the presence of 0.2 μ*M* ryanodine and 2.0, 4.0, and 8.0 m*M* extracellular Ca^{2+}. Tetanic stimulation was continued for 5 s because longer tetani might cause damage to the muscle and would increase aequorin consumption. Measurements were made at the end of the 5-s tetanus. Upper trace, aequorin light signal; Lower trace, isometric contraction.

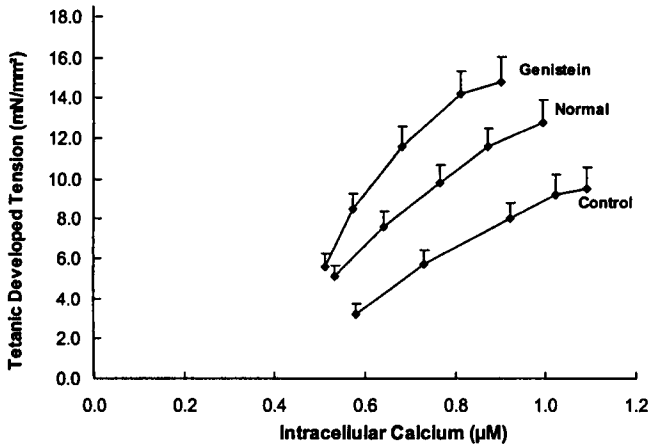

Figure 4. Graph showing force-$[Ca^{2+}]_i$ relation as recorded in tetanized papillary muscles isolated from adult Wistar rats. Normal, rat papillary muscles without ischemia ($n = 8$); Control, postischemic rat papillary muscles without genistein ($n = 7$); Genistein, postischemic rat papillary muscles in the presence of genistein (2 μ*M*; $n = 8$). Peak tetanic isometric force is plotted against the value of intracellular Ca^{2+} during the tetanic plateau phase of aequorin light signal. Tetanus was elicited in the presence of 0.2 μ*M* ryanodine and 1.0, 2.0, 4.0, 8.0, and 12.0 m*M* extracellular Ca^{2+}.

genistein-treated group. The Hill coefficient was calculated as 7.0 ± 1.3 in the normal group, 7.9 ± 1.4 in the control group, and 6.7 ± 1.0 in the genistein-treated group.

Effect of Genistein on Protein Levels of TnI. After the ischemia-reoxygenation cycle in fluorocarbon immersion, protein levels of TnI were measured in nonischemic papillary muscles and postischemic muscle preparations (five of each) from control and genistein-pretreated groups. Samples of postischemic papillary muscles were obtained after 60 min of reoxygenation in fluorocarbon. TnI protein immunoreactivity was decreased in control papillary muscles after ischemia compared with muscles in the presence of genistein (Fig. 5). The representative Western blots of TnI and immunoreactivity of TnI protein normalized to cyclophilin A in nonischemic papillary muscles, control, and genistein-pretreated groups are presented in Figure 5.

Discussion

In the present study, we demonstrated that genistein, a tyrosine kinase inhibitor, attenuated the depressed postischemic myocardial function in isolated papillary muscles. This beneficial effect on the ischemia-reperfusion cycle was associated with an increase in the Ca^{2+} sensitivity of the myofilaments.

The simulated ischemia model, which has been successfully used in our laboratory (15–17), was produced by immersing the papillary muscle into hypoxic fluorocarbon due to the nonpolar structure of the fluorocarbon. We found that a decrease in developed tension in postischemic papillary muscles compared with controls was similar to a reduction in developed pressure in isolated stunned perfused hearts (2, 18). Thus, the use of isolated papillary muscles in the fluorocarbon-simulated ischemia model offers a novel and useful approach to investigate excitation-contraction coupling in postischemic myocardium (15–17).

It is widely accepted that postischemic reperfusion injury causes cardiac dysfunction, known as stunning (19–21). Although there is a general consensus that free radicals (22), damage of extracellular collagen matrix (23), and impaired myocardial energy production and use (24) are related to postischemic myocardial dysfunction, the precise mechanism is still controversial. The basis for depressed postischemic myocardial function may be related to a reduction of the contractile machinery responsiveness to Ca^{2+}. Reported data from our laboratory (1, 18) and others (25–27) have shown that myocardial stunning is at least partially due to a decrease in myofilament Ca^{2+} sensitivity. In the present study, we demonstrated that both myocardial contractility and Ca^{2+} sensitivity of myofilaments were reduced in postischemic cardiac muscle. The mechanism un-

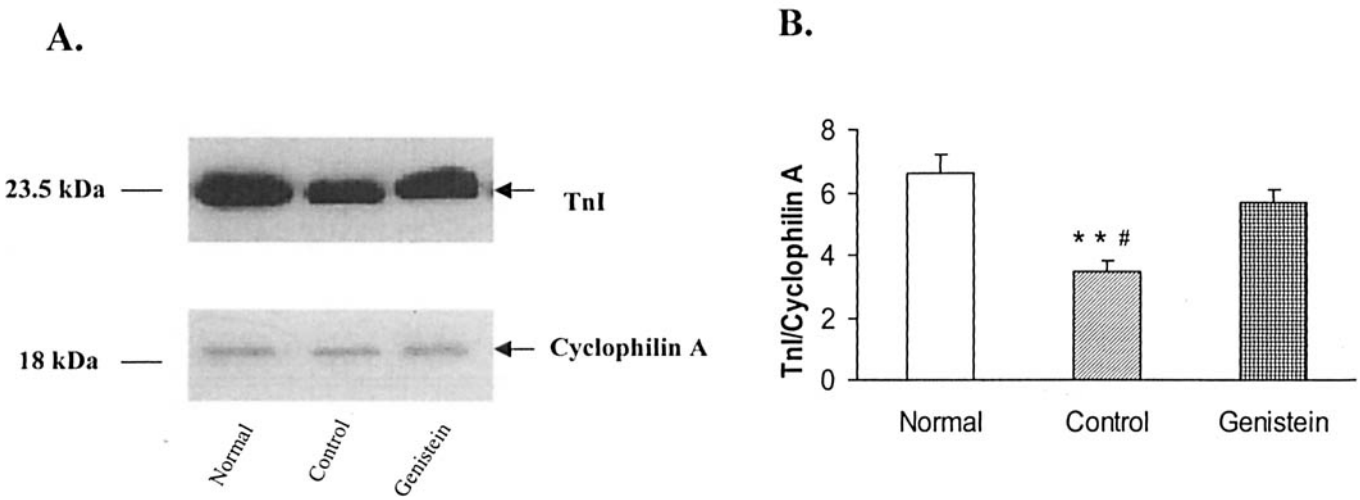

Figure 5. Protein expression of TnI in rat papillary muscles ($n = 5$) at the phase of postischemia was assessed by the Western blot (A) and densitometric analysis of TnI/Cyclophilin A (B). Normal, rat papillary muscles without ischemia; Control, rat postischemic papillary muscle without genistein; Genistein, rat postischemic papillary muscle in the presence of genistein (2 μ*M*). $^{**}P < 0.01$ vs. Normal; $^{\#}P < 0.05$ vs. Genistein.

derlying the decreased Ca^{2+} responsiveness remains unknown at present.

In the present study, the alteration of TnI on the immunoblots could reflect loss of the TnI protein, partial proteolysis of the TnI, or changes in the TnI phosphorylation. Reported studies by Marban's group (28, 29) suggested that postischemic stunning is associated with catabolism of TnI. Previous experiments indicated that phosphorylation of TnI by a cAMP-dependent protein kinase or phosphorylase kinase can decrease Ca^{2+} sensitivity (30). With isolated rat heart myofibrils, Wattanapermpool *et al.* (31) demonstrated a significant role for TnI in mediating the decrease in myofilament Ca^{2+} sensitivity associated with PKA-dependent phosphorylation. Densitometric analysis of myofibrillar proteins found a new protein band appearing in rat myofibrils after complete global ischemia (9). ^{32}P-ATP autoradiogram showed that this new band is a phosphorylated TnI fragment induced by cAMP-dependent protein kinase. Strang *et al.* (8) also showed that both isoproterenol and PKA treatment in rat cardiac myocytes reduced the Ca^{2+} sensitivity during isometric contraction. It has been accepted that ischemia can produce high concentrations of catecholamines in the myocardium due to exocytotic and nonexocytotic release from adrenergic nerve endings, and impaired reuptake mechanisms (32–34). Reoxygenation injury may also occur after a sudden transmembrane Ca^{2+} influx mediated via increased catecholamine release (35). Stimulation of α-adrenoreceptors (7, 36) and β-adrenoreceptors (30) by an increased concentration of catecholamines during the period of ischemia-reperfusion activates the ERK cascade (the common pathway of activated protein kinases) (37). Activated ERKs in cardiac myocytes by β-adrenoreceptor agonists, including isoproterenol, have been demonstrated by Bogoyevich (37) and Yamazaki *et al.* (11). A previous study (23) demonstrated that tyrosine kinases are responsible for activation of ERKs by isoproterenol and found that activation was suppressed completely when cardiomyocytes were pretreated with a broad-spectrum tyrosine kinase inhibitor, genistein. This suggests that the tyrosine kinase is involved in isoproterenol-induced activation of ERKs in cardiac myocytes.

The present findings indicate that genistein ameliorated postischemic dysfunction by enhancing the myofilament sensitivity to Ca^{2+}. The increase of myofilament Ca^{2+} sensitivity by additional genistein might result from diminished phosphorylation of TnI, which is reflected by partially restoring the TnI protein levels in postischemic papillary muscles. Although studies, both previous (38) and more recent (39), demonstrated that exceeding 0.2 mm in muscle preparation diameter causes core hypoxia, the data presented here are comparable because all results were collected under the same experimental conditions in both control and genistein-treated groups. The present study was conducted at a low stimulating frequency and a 30°C temperature. Additional experiments are required to investigate the phosphorylated status of TnI during the ischemia-reperfusion sequence under physiological conditions.

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