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Antioxidant Therapy Attenuates JNK Activation and Apoptosis in the Remote Noninfarcted Myocardium after Large Myocardial Infarction

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We hypothesized that the concomitant occurrence of increased oxidative stress, JNK activation, and myocyte apoptosis in the remote myocardium (RM) following a large myocardial infarction (MI) are causally related. Three days following coronary ligation, rats were randomized to treatment with probucol and PDTC (MI-T) or vehicle (MI). Control rats (C) underwent sham operation. At 7 weeks, TBARS assay showed increased level of lipid-peroxidation within the RM in the MI group vs C, which was completely inhibited in the MI-T group. Similarly, Western blot analysis showed a twofold increase in p-JNK in the MI group, vs C, which was attenuated in MI-T, a result confirmed by a JNK-kinase activity. Furthermore, apoptosis was increased within the RM in MI vs C, while this was inhibited in MI-T. We conclude that long-term antioxidant therapy with probucol and PDTC attenuates oxidative stress, JNK activation, and myocyte apoptosis within the RM after large MI. © 2001 Academic Press

Key Words: **oxidative stress; antioxidants; JNK; apoptosis; heart failure.**

Increased activity of stress-activated protein kinase SAPK/JNK (c-Jun NH_2-terminal kinase), increased myocyte apoptosis, and increased oxidative stress were recently shown to occur concomitantly within the remote noninfarcted myocardium (RM) following a large myocardial infarction in rats (1).

JNK activity plays an important role in the signal-transduction leading to various cellular responses, including hypertrophy (2, 3), proliferation (4) and apoptosis (5–7). JNK activation within the RM may therefore be of significant relevance to the myocardial remodeling which takes place in the RM after myocardial infarction. This remodeling process is characterized by myocyte elongation and enlargement, while at the same time, there is evidence for significant loss of cardiac myocytes (8), most likely induced by apoptosis (9).

Since oxidative stress is a powerful activator of JNK (10–13), and JNK activation is an important signaling step leading to cardiac myocyte apoptosis (14, 15), we tested the hypothesis that the co-existence of oxidative stress, JNK activation and apoptosis within the RM is causally related.

We show in this study, for the first time, that an effective long-term antioxidant therapy concomitantly attenuates JNK activation and cardiac apoptosis in the RM after a large myocardial infarction in rats.

MATERIALS AND METHODS

Animal model and study groups. Antero-lateral myocardial infarction (MI) was produced by coronary ligation (16) in male Sprague-Dawley (SD) rats (180–200 g). Control animals underwent sham operation. Three days later the infarcted rats were randomly divided into two groups: One group received active treatment (MI-T), probucol 37 mg/kg/week and pyrrolidine dithiocarbamate (PDTC) 280 mg/kg/week, in three divided doses into the peritoneal cavity (i.p.); the other received vehicle only, i.p., three times per week (MI). Control rats (C) were also given vehicle i.p. three times per week. All three groups were provided with food and water ad libitum.

Determination of infarction size. Two-dimensional Echo imaging was performed using an Acuson (Mountainview, CA) Sequoia C256 unit, equipped with a 13.2-MHz linear array transducer. Ejection fraction and myocardial infarction size was estimated as detailed previously (17).

Tissue preparation. After 7 weeks the rats were sacrificed, the hearts excised and placed on ice, the myocardium flushed with ice-cold Krebs buffer via the aortic root and the right and left ventricles separated and weighed. The left ventricle was sliced into segments along the short axis. One segment from the midventricle was fixed in 4% phosphate buffered formalin and embedded in paraffin. The remainder of the heart slices were divided into three parts and stored under liquid nitrogen for further analysis. The three parts were defined as follows: (a) the infarcted myocardium, which was macroscopically mostly a white scar tissue, (b) border zone, which was 2 mm into the scar and 2 mm into the noninfarcted tissue, (c) the remote noninfarcted myocardium which defined as the unscared tissue between the borderzones, at least 2 mm away from the macro-

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scopic scar. Histological examination with H&E staining showed that 2 mm beyond the scar tissue, the myocardium appeared normal.

Thiobarbituric acid reacting substances assay. Lipid peroxide content in myocardium was determined by using the thiobarbituric acid reactive substances (TBARS) method as described previously (18).

JNK kinase activity assay. Aliquots of 0.5 ml containing 0.5 mg of protein extracts in Triton X-100 lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 0.1 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA) were incubated with 1 μg anti-JNK antibody overnight at 4°C. Subsequently, 15 μl of a Protein-G-agarose bead suspension was added and the slurries were incubated with mixing for 1 h at 4°C. The immunocomplexes were washed three times with lysis buffer and once with kinase assay buffer (100 mM Tris–HCl, pH 7, 40 mM Magnesium Acetate, 0.4 mM Na_3VO_4, 0.4 mM EGTA), then incubated with c-$Jun_{1\text{-}79}$, the substrate for JNK (Santa Cruz Biotechnology, Santa Cruz, CA), as well as [γ-^{32}P]ATP. Protein in the kinase reaction were resolved by SDS–PAGE electrophoresis and subjected to autoradiography.

Western blot. The myocardium was homogenized in PBS containing a protease inhibitor cocktail, the homogenate subjected to SDS–PAGE (Fig. 1C demonstrates equal protein loading as described previously (1). The membranes were probed using mouse monoclonal anti-p-JNK antibody and mouse monoclonal anti-JNK1 antibody (Santa Cruz, CA).

In situ TdT-UTP nick-end labeling (TUNEL staining). TUNEL staining was performed as described previously (1). Briefly, after deparaffinization and rehydration, 5 μm thick tissue sections were incubated in PBS, containing 0.1% saponin and 1 mM EGTA for 30 min. Subsequently, sections were covered with a solution containing 0.1 U/μl of terminal deoxynucleotidyl transferase, 2.5 mM $CoCl_2$, 0.2 M Sodium cacodylate, 25 mM Tris–Cl, 0.25% BSA and 0.5 nM 2′-biotin-16-dUTP. Sections were incubated in this solution for 45 min at 37°C in a humidified chamber. After incubating with HRP-Avidin (0.5 mg/ml in PBS) 30 min at 37°C, the sections were stained with diaminobenzidine. This process yields deep brown staining of positive nuclei and clear light brown staining of myocyte cytoplasm; nuclei were counterstained with methyl green and yielded green negative nuclei (representive color figure in reference (1)). Four sections of each sample, each cut at 20 μm intervals from the paraffin block, were used for staining. All of the stained areas were counted. Only tissue that was at least 2 mm from the infarct margins was considered to be remote myocardium and included in the analysis. Results were presented as the number of apoptotic nuclei within myocytes per 10^5 total nuclei counted.

Statistical analysis. Data are presented as mean ± SEM and ANOVA used for analysis. A P value < 0.05 was considered significant.

RESULTS

Infarct size and area ejection fraction were similar in both infarct groups when treatment was initiated (day 3), infarct size = 52.2 ± 5.7 in MI vs 57.6 ± 3.6 in MI-T, $n = 3$, $P = 0.48$; EF = 28.8 ± 4.8 in MI vs 34.3 ± 5.7 in MI-T, $n = 3$, $P = 0.5$. This assures that the RM in both infarct groups, treated and untreated, were exposed to similar degree of hemodynamic strain post infarction.

TBARS assay was used to compare the level of lipid-peroxidation within the RM from each group at 7 weeks. There was a significant increase in TBARS within the RM from the untreated infarcted hearts (in MDA nmol/g protein); 157 ± 26 vs control 83 ± 4, $P < 0.05$. This was completely inhibited in the RM from the treated infarcted rats (79 ± 4), demonstrating the antioxidant effect of the active treatment protocol.

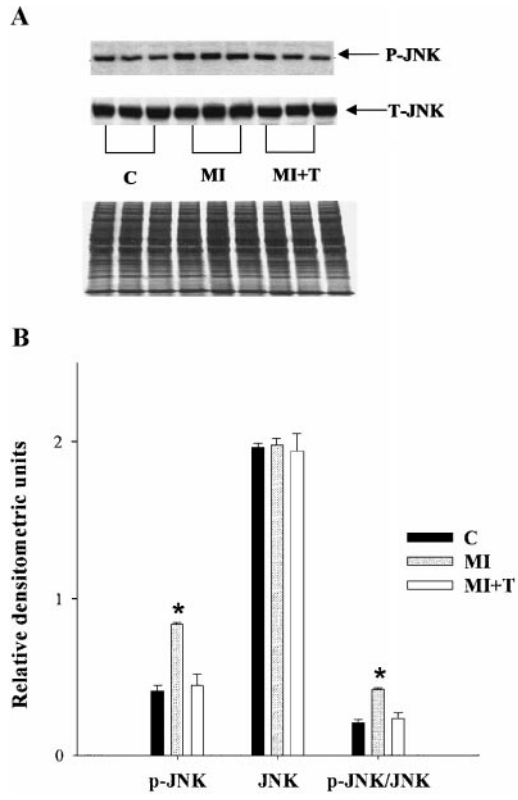

FIG. 1. Activation of JNK was determined by Western blot. (A) The upper panel shows the results of blotting with mouse monoclonal anti-p-JNK antibody on samples from the RM in C, control rats, MI, infarcted rats treated with vehicle only, and MI + T, infarcted rats treated with probucol and PDTC. The middle panel shows the results of blotting with rabbit anti-human-JNK1 antibody. The lower panel shows Coomassie blue staining, demonstrating equal protein loading on the SDS–PAGE gel. (B) This bar graph displays the numeric results of the top two panels when quantified by densitometry. The optical density value of p-JNK and JNK and the ratio of p-JNK/JNK were expressed as mean ± SE, ($n = 3$), *$P < 0.05$ compared with C and MI + T.

JNK activity was evaluated by Western blot analysis. Using specific antibody to phosphorylated JNK (p-JNK), representing the active form of JNK, a two-fold increase in p-JNK was demonstrated within the RM from the infarcted untreated hearts vs control hearts. This increase in p-JNK was blocked in the RM from the treated infarct group (Fig. 1A). There was no significant change in total-JNK (Fig. 1B). As previously demonstrated in this model (1), the result of the Western blot analysis was confirmed by a JNK kinase activity assay, using GST-c-Jun as a substrate for activated JNK (Fig. 2A).

TUNEL staining was used as a measure of apoptosis within the myocardium. There was a significant increase in TUNEL positive myocyte in the RM of the

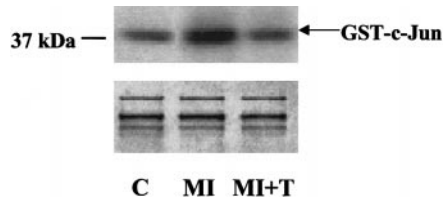

FIG. 2. JNK activation determined by kinase activity assay. Upper panel shows results of JNK activity on protein samples from the remote non-infarcted myocardium from: C, control; MI, infarcted rats treated with vehicle only; MI + T, infarcted rats treated with probucol and PDTC. Lower panel shows Coomassie blue staining, demonstrating equal protein loading on the SDS–PAGE gel.

untreated infarct group (19.7 ± 1.2 myocytes per 10^5 total nuclei counted) compared to control (7.4 ± 0.9, $P < 0.05$). This increase was significantly attenuated in the RM of the treated infarct group (12.1 ± 1.4). These results were confirmed with DNA electrophoresis, showing typical DNA laddering in the DNA extracted from the MI group, while it was barely detectable in the C and MI-T groups (Fig. 3).

DISCUSSION

This study shows that long term therapy with probucol and PDTC, which effectively inhibits oxidative stress in the RM, attenuates the activation of stress-activated protein kinase SAPK/JNK and reduces cardiac myocyte apoptosis within the RM.

Oxidative Stress within the RM

This study substantiates previous reports which show evidence of increased oxidative stress within the RM after large myocardial infarction (1, 17, 19, 20). Furthermore, it shows that long-term treatment with the antioxidants probucol and PDTC can effectively prevent the increased oxidative stress.

Importantly, the initial infarct size was similar in both infarct groups, assuring similar hemodynamic strain on the RM in both groups at baseline. Furthermore, the antioxidant drugs chosen, are both well established antioxidants that cross cell membranes easily, which allows them to scavenge oxygen free radicals formed within cells. Their effectiveness in protecting the myocardium from oxidative injury has previously been clearly demonstrated (21, 22). In addition, we have shown that when these drugs were given to healthy control rats for two weeks, they had no significant effect on heart rate and blood pressure (data not shown), suggesting that their effects observed in this study are primarily mediated via their antioxidant properties.

In this study TBARS assay was used to measure the degree of lipid-peroxidation within the RM. The validity of this method to assess the level of oxidative stress within the myocardium in this experimental model is strongly supported by recent studies by Hill *et al.* (19, 20) who showed a close correlation between changes in TBARS and several other sophisticated indicators of oxidative stress in the myocardium.

Oxidative Stress and JNK Activation in the Heart

Oxidative stress is known to activate stress-activated protein kinase SAPK/JNK (10–13). *In vitro,* hydrogen peroxide causes activation of JNK in cardiac myocytes (10) and *in vivo,* myocardial ischemia followed by reperfusion, a classic model of oxidative stress, causes activation of JNK in the heart (23–25). Furthermore, oxidative stress has been also associated with an increase in JNK activity within the RM several weeks after a large myocardial infarction (1). The novel finding of this study is that long-term treatment with antioxidants did not only effectively inhibit lipid-peroxidation within the RM of the treated animals, but simultaneously attenuated the increase in JNK activation seen in the RM of untreated rats. This suggests, but does not prove, a direct cause and effect relationship between oxidative stress and JNK activation in the heart. However, further support of this concept is provided by a recent report, showing that short term treatment with carvedilol, a β-adrenergic receptor blocker with antioxidant properties, attenuated JNK activation during acute myocardial ischemia-reperfusion injury, while propranolol another β-adrenergic receptor blocker without significant free radical scavenging activity, had no effect (25).

Other stimuli have also been noted to upregulate JNK activity in cardiac myocytes. These include

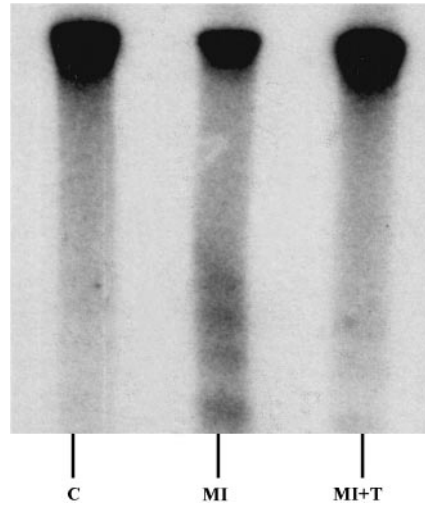

FIG. 3. Apoptosis was inhibited by antioxidants in the remote noninfarcted myocardium. This figure represents agarose gels of DNA isolated from a control heart, C, and the remote noninfarcted myocardium in: MI, infarcted rats treated with vehicle only and MI + T, infarcted rats treated with probucol and PDTC.

angiotensin-II (26) and mechanical stretch (27). However, it is of interest that both of these stimuli are associated with increased oxygen free radical production (28, 29) that may therefore play a role in the signaling pathway leading to the activation of JNK in these instances.

Oxidation-Induced JNK Activation and Cardiac Myocyte Apoptosis

In addition to activation of JNK, oxidative stress has also been shown to be a strong inducer of cardiac myocyte apoptosis *in vitro* (14, 30) as well as *in vivo* (31). Thus the question arises, is there a causative link between the observed JNK activation and the cardiac myocyte apoptosis? While the current study only provides suggestive evidence to support this hypothesis, several recent *in vitro* studies have provided more convincing direct data that this may indeed be the case (14, 15). Turner *et al.* showed in an *in vitro* model that hydrogen peroxide induced apoptosis, which was associated with an increase in JNK activity, but could be blocked by transfection of a dominant negative mutant of JNK (14). Furthermore, Yue *et al.* showed recently that inhibition of JNK activity attenuated significantly ischemia/reoxygenation-induced apoptosis in cultured myocytes and in isolated perfused hearts (15). Thus, while the mechanism of cardiac myocyte apoptosis within the RM after myocardial infarction is likely to be multifactorial, activation of JNK secondary to increased oxidative stress may play a significant role.

However, it should also be realized that JNK activation plays an important role in signal-transduction leading to various cellular responses other than apoptosis, including cell hypertrophy (2, 3) and cell proliferation (4). This is quite relevant to myocardial remodeling within the RM, which in addition to myocyte loss (8, 9) is characterized by myocyte hypertrophy due to elongation and enlargement of individual myocytes. In addition, recent data suggest that cardiomyocyte proliferation can also take place in a failing adult heart (32). Thus, whether JNK activation in an individual myocyte eventually leads to hypertrophy, proliferation or apoptosis, is likely to be determined by multiple factors. These may include the degree of activation, whether the activation is short or sustained (6), and by complex interactions between the JNK pathway and other signaling mechanisms within the cell, as well as the microenvironment that the cell is surrounded by.

Conclusion

Long-term treatment with probucol and PDTC decreases oxidative stress and significantly attenuates JNK activation in the remote non-infarcted myocardium after a large myocardial infarction. Treatment with antioxidants may have future role in treatment of congestive heart failure.

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