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Biochemical Pharmacology 62 (2001) 483–493

Biochemical Pharmacology

Inhibition of stress-activated protein kinase in the ischemic/reperfused heart: role of magnesium tanshinoate B in preventing apoptosis

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Received 29 June 2000; accepted 16 November 2000

Abstract

The activation of stress-activated protein (SAP) kinase may lead to an induction of apoptosis that is responsible for part of the cardiomyocyte death in reperfusion injury. The objective of the present study was to investigate the mechanism by which magnesium tanshinoate B (MTB), a bioactive compound isolated from Danshen, prevents apoptosis in cardiomyocytes in the ischemic/reperfused heart. Isolated adult rat hearts were perfused by the Langendorff mode with medium containing MTB prior to the induction of normothermic global ischemia. At the end of the 30-min ischemic period, the heart was reperfused with the same medium with or without MTB for an additional 20 min. In the MTB-treated ischemic/reperfused heart, the number of apoptotic nuclei was reduced by 2.5-fold in comparison to that in untreated ischemic/reperfused controls [23 ± 4 vs 57 ± 7 (mean ± SD) TUNEL-positive cells, respectively, $N = 3–4$, $P < 0.001$]. SAP kinase activity was elevated 1.7-fold in ischemic/reperfused rat hearts [35.6 ± 3.8 vs 21.2 ± 3.3 (control) (mean ± SEM) relative densitometric units, $N = 4–6$, $P < 0.05$]. Treatment with MTB abolished this elevation in SAP kinase activity (25.0 ± 5.2 relative densitometric units), which was also decreased by 40% in the nucleus. When the heart was subjected to ischemia alone, there was no significant change in SAP kinase activity in the presence or absence of MTB. MTB did not appear to affect the p38 mitogen-activated protein kinase activity in this model system. In conclusion, MTB was shown to have cardioprotective activity against apoptosis, probably through the inhibition of SAP kinase activity.

Keywords: Apoptosis; Ischemia; Protein kinases; Protein phosphorylation; Reperfusion

1. Introduction

Over the last decade, a new superfamily of protein kinases activated by mitogens and growth factors was recognized. Broadly referred to as the MAP kinases, they include the extracellular signal-regulated protein kinases, the SAP kinases and p38 MAPK [1]. Cellular stresses, e.g. ischemia/reperfusion, heat shock, hyperosmotic conditions, UV irradiation, protein synthesis inhibitors such as anisomycin, as well as the proinflammatory cytokines like tumor necrosis factor-α, have been reported to activate the latter two MAP kinase homologues, namely SAP kinase and p38 MAPK [1].

The activation of SAP kinase and p38 MAPK may play a critical role in the genetic response of components of the cardiovascular system to disease states such as ischemia, atherosclerosis, heart failure, and restenosis [2]. The disease process can produce oxygen free radicals and factors (e.g. cytokines), which can activate the SAP kinases [3–5] in addition to causing oxidation of cellular components, oxidation of the membrane (causing leakage), and modification of membrane receptors [6]. The activated SAP kinases can then translocate to the nucleus [4], where they may induce a host of immediate early genes [7], leading to apoptosis. Activation of the SAP kinase cascade is one of the mecha-

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Abbreviations: JIP, JNK-interacting protein; MAP, mitogen-activated protein; p38 MAPK, p38 MAP kinase; MTB, magnesium tanshinoate B; PARP, poly(ADP-ribose) polymerase; PMSF, phenylmethylsulfonyl fluoride; SAP, stress-activated protein; and TUNEL, terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick-end labeling.

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PII: S0006-2952(01)00686-4

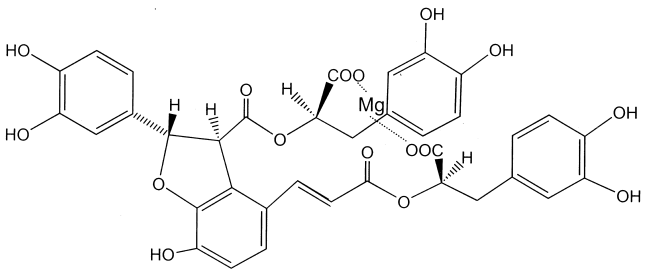

Fig. 1. Structure of magnesium tanshinoate B (MTB; magnesium lithospermate B).

nisms for the induction of apoptosis [8–11]. Recent evidence suggests that a proportion of the cardiomyocytes preferentially undergoes apoptosis following myocardial ischemia/reperfusion [12,13]. In ischemic/reperfusion injury, a sustained activation of SAP kinases has been reported [4,5,14]. This may account for the apoptotic death of cardiomyocytes. Any attenuation of the activation of SAP kinase may, in turn, reduce the loss of cardiomyocytes, thus producing a partial offset to reperfusion injury.

Danshen (*Radix Salvia miltiorrhizae*) has been used in the treatment of cardiovascular disease in China [15–17] and has been shown to lower blood pressure [18–21]. In the isolated rat heart, Zhou and Ruigrok [21] showed that Danshen had a negative inotropic effect and increased coronary blood flow. Furthermore, Danshen-treated hearts had better post-ischemic reperfusion recovery of ventricular developed pressure and less contracture than untreated hearts [21]. Clinically, in a double-blind study using phenolphthalein as a placebo, Danshen was reported to be effective in the treatment of coronary heart disease [22]. Other studies have revealed that Danshen was able to dilate the rat aorta in an endothelium-dependent manner [20,23]. This vasodilatory action of Danshen might be due, in part, to the action of one of its active ingredients, tanshinone II-A. Tanshinone II-A is a naturally occurring calcium antagonist and can cause coronary and peripheral vasodilation by reducing the influx of calcium into myocardial and smooth muscle cells [21]. Another active component of Danshen is MTB (Fig. 1), also known as magnesium lithospermate B. Infusion of MTB into the post-ischemic rabbit heart has been shown to reduce damage to the myocardium when compared with the saline-treated control [24]. Futhermore, intravenous injection of MTB (30 mg/kg) into rats resulted in a decrease in blood pressure with no changes in the heart rate [20]. However, the mechanisms of these MTB actions are largely unknown.

In the present study, the effect of MTB on ischemia/reperfusion-induced apoptosis was investigated. Our results demonstrated that MTB can protect the ischemic/reperfused heart against apoptotic cell death. Further investigations revealed that MTB can directly inhibit SAP kinase activity and its translocation into the nucleus. This may represent one of the mechanisms by which MTB exerts its cardioprotective effect.

2. Materials and methods

2.1. Materials

MTB was isolated from *Radix Salvia miltiorrhizae* by standard chromatographic techniques [24], and its purity was determined by HPLC to be $>$ 98%. SAP kinase and p38 MAPK assay kits, and rat-specific polyclonal antibody against cleaved PARP (D214) were purchased from New England Biolabs and Cell Signaling Technology, respectively. The expression plasmid pGEX-GST-ATF2 (19–96) was a gift from Dr. J. Silvio Gutkind of NIDR, NIH; recombinant GST-ATF2 (19–96) was prepared according to the protocols of Amersham Pharmacia Biotech. All other chemicals were of reagent grade and were obtained from the Sigma Chemical Co. or BDH Chemicals. For heart perfusion, male Sprague–Dawley rats (200–250 g) were used. All experimental protocols involving the use of animals were approved by the Committee for the Use of Live Animals for Teaching and Research of The University of Hong Kong.

2.2. Heart perfusion

Perfusion of the rat heart was performed as previously described [25]. Male Sprague–Dawley rats (200–250 g) were anaesthetized by i.p. injection of sodium pentobarbitone (7 mg/100 g body weight) and heparin (20 IU/100 g body weight). Hearts were then excised and perfused in a Langendorff apparatus with Krebs-Henseleit buffer containing 120 mmol/L of NaCl, 25 mmol/L of $NaHCO_3$, 5.5 mmol/L of dextrose, 4.76 mmol/L of KCl, 1.2 mmol/L of magnesium sulfate, 1.2 mmol/L of KH_2PO_4, and 1.27 mmol/L of $CaCl_2$, saturated with 95% O_2/5% CO_2 for 10 min at 37°. This was followed by perfusion with fresh Krebs-Henseleit buffer containing MTB for 20 min. Subsequently, normothermic global ischemia was induced by stopping the flow of buffer for 30 min (unless stated otherwise) while maintaining the temperature at 37°. At the end of the ischemic period, the hearts either were processed immediately or were reperfused with the same medium containing MTB for an additional 120 min, unless stated otherwise. Perfused hearts were processed as described below or were frozen in liquid nitrogen and stored at –70° until used.

2.3. In situ *labeling of DNA fragments (TUNEL)*

DNA fragmentation was detected *in situ* by using TUNEL in the perfused heart [26]. After perfusion, hearts were fixed in phosphate-buffered 4% paraformaldehyde at 4° overnight prior to embedding in paraffin. Embedded tissue (5 μm sections) was adhered to slides that had been pretreated with Vectabond^{TM} (Vector Laboratories Inc.). Paraffin was removed from sections by immersing in xylene; rehydrated through 100, 96, 80, 70, and 0% ethanol;

and incubated in 10 mmol/L of Tris–HCl (pH 8.0) for 5 min. The nuclei of the tissue sections were stripped of proteins by incubation with 20 μg/mL of proteinase K (Sigma) for 20 min at 37°. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol for 5 min at room temperature, and sections were washed several times with water and PBS. DNA fragments in sections were labeled with 3 μM biotin-conjugated dUTP (Roche Molecular Biochemicals) and 30 U/mL of TdT (Life Technologies) in a buffer containing 30 mmol/L of Tris, 140 mmol/L of sodium cacodylate, pH 7.2, and 1 mmol/L of cobalt chloride, for 90 min at 37° in a humidified chamber. The reaction was terminated by transferring the slides to a bath containing 300 mmol/L of NaCl and 30 mmol/L of sodium citrate (pH 8) for 15 min at room temperature. After washing with PBS, the slides were blocked with 2% BSA for 10 min at room temperature. Sections were then processed by standard immunoperoxidase techniques using diaminobenzidine as chromogen. The sections were then counter-stained with Mayer's hematoxylin, dehydrated, and mounted using DPX mountant (BDH). Slides incubated without TdT or treated with DNase I were used as negative and positive controls, respectively.

The number of TUNEL-positive cardiomyocytes was counted, under a light microscope at a magnification of 400X, from three different cross-sections of the left and right ventricle of each perfused heart. A minimum of 10 fields with a total nuclei number of 2937 ± 118 (mean ± SD) was counted in each cross-section of the heart.

2.4. Preparation of tissue extract

An extract of the perfused heart was prepared as described previously [25]. Briefly, frozen rat hearts were first pulverized using a Bio-Pulverizer (Biospectronic) and then were thawed on ice prior to homogenization using a Polytron PT 2100 (Kinematica AG, Littau-Lucerne) (2 × 30 sec), in a buffer containing 20 mmol/L of Tris (pH 7.4), 150 mmol/L of NaCl, 1 mmol/L of EDTA, 1 mmol/L of EGTA, 2.5 mmol/L of sodium pyrophosphate, 1 mmol/L of β-glycerophosphate, 1 mmol/L of sodium orthovanadate, 2.1 μmol/L of leupeptin, 1 mmol/L of PMSF, and 1% Triton X-100. The homogenate was centrifuged at 100,000 rpm (417,000 *g*) for 15 min at 4° in a Beckman Optima-TLX Tabletop ultracentrifuge, and the supernatant was used for the determination of SAP kinase activity. The amount of protein in the supernatant was determined according to the method of Bradford [27].

2.5. Measurement of SAP kinase and p38 MAPK activity

SAP kinase activity was determined using a SAPK/JNK assay kit (New England Biolabs). Briefly, the protocol involved a 15-hr overnight incubation at 4° of the cytosolic extract with 2 μg of agarose-immobilized GST-c-Jun (1–89). This mixture was then washed twice with the incubation buffer, followed by two additional washes with kinase buffer [25 mmol/L of Tris (pH 7.5), 5 mmol/L of β-glycerophosphate, 0.1 mmol/L of sodium orthovanadate, 2 mmol/L of dithiothreitol, and 10 mmol/L of $MgCl_2$]. For the phosphotransferase reaction, 100 μmol/L of ATP was added to initiate the enzyme reaction, which was allowed to proceed for 30 min at 30°. After terminating the kinase reaction using SDS sample buffer, the samples were separated by SDS–10% PAGE, electrotransferred onto a nitrocellulose membrane, and analyzed by western immunoblotting. A polyclonal phosphospecific antibody that recognized the phosphorylated Ser-63 on GST-c-Jun (1–89) was used as the primary antibody, and the blot was developed with ECL reagent (Amersham Pharmacia Biotech). The resulting autoradiogram was analyzed using the Gel Doc 1000 and the Multi-Analyst® software version 1.1 program from Bio-Rad (Hercules).

p38 MAPK activity was determined using a p38 MAPK assay kit (New England Biolabs). Briefly, the protocol involved immunoprecipitating the active p38 MAPK using a monoclonal phospho-specific antibody, followed by the phosphotransferase reaction (30 min at 30°) using GST-ATF2 (19–96) as substrate. At the end of the incubation period, the samples were separated by SDS–PAGE and analyzed by western immunoblotting as described above, using a polyclonal phosphospecific antibody that recognized phosphorylated Thr-71 on GST-ATF2 (19–96).

2.6. Subcellular fractionation

Subcellular fractionation of the perfused hearts was performed at 4° as previously described [4], with minor modifications. Frozen rat hearts were first pulverized using a Bio-Pulverizer and then were thawed on ice prior to homogenization using a Polytron PT 2100 (2 × 30 sec), in 2 vol. of a buffer (Buffer A) containing 0.32 mol/L of sucrose, 10 mmol/L of Tris–HCl (pH 7.4), 1 mmol/L of EGTA, 1 mmol/L of EDTA, 5 mmol/L of sodium azide, 10 mmol/L of β-mercaptoethanol, 20 μmol/L of leupeptin, 0.15 μmol/L of pepstatin A, 0.2 mmol/L of PMSF, 50 mmol/L of sodium fluoride, 1 mmol/L of sodium orthovanadate, and 10 mmol/L of sodium pyrophosphate. The homogenates were mixed with an additional 2 vol. of Buffer A and centrifuged (1000 *g* for 10 min) to obtain the nuclear pellets. This pellet was washed once with Buffer A to obtain the nuclear fraction, while the supernatant was centrifuged (100,000 *g* for 60 min) to obtain the cytosolic fraction. The nuclear fraction was solubilized in Buffer B [1% Triton X-100, 150 mmol/L of NaCl, 10 mmol/L of Tris–HCl (pH 7.4), 1 mmol/L of EGTA, 1 mmol/L of EDTA, 20 μmol/L of leupeptin, 0.15 μmol/L of pepstatin A, 0.2 mmol/L of PMSF, 50 mmol/L of sodium fluoride, 0.2 mmol/L of sodium orthovanadate, 10 mmol/L of sodium pyrophosphate] and centrifuged at 15,000 *g* for 30 min. The nuclear extract (supernatant) was stored at –70° until used.

2.7. Statistical analysis

All values are expressed as means ± SEM. Comparisons between groups were assessed by one-way ANOVA with Tukey's post hoc-analysis. Spearman rank order correlation coefficient was calculated with SPSS for Windows (Version 10) (SPSS Inc.). Statistical significance was defined as a value of $P < 0.05$.

3. Results

3.1. Effect of MTB on apoptosis in the isolated ischemic/reperfused heart

Since MTB has been shown to reduce myocardial damage in the post-ischemic rabbit heart [24], its effect on cardiomyocyte apoptotic cell death was first investigated. Apoptosis in cardiomyocytes was assessed by counting the number of TUNEL-positive cells. TUNEL positivity was characterized by focal nuclear staining [26]. As shown in Fig. 2, in apoptotic cells, the nuclear and cell membrane integrity remained intact. In our model system of ischemia/reperfusion, there was no difference in the number of TUNEL-positive nuclei between the left and right ventricles. The reaction product was dark brown, and there was minimal background. The number of TUNEL-positive nuclei in the heart was increased significantly (by 2.5-fold) after ischemia/reperfusion (Figs. 2, c and d, and 3). In contrast, when the isolated rat heart was perfused in the presence of 1 μg/mL of MTB (Fig. 2, a and b), the number of TUNEL-positive nuclei after ischemia/reperfusion was comparable to that of the non-ischemic/perfused heart (Fig. 2, e and f). A significant 2.5-fold reduction in the number of TUNEL-positive nuclei was observed in the MTB-treated ischemic/reperfused heart (23 ± 4 nuclei, mean ± SD) when compared with the untreated ischemic/reperfused control (57 ± 7 nuclei) (Fig. 3).

Apoptosis was also assessed by the detection of PARP cleavage in the cell. The proteolytic cleavage of the 116 kDa PARP into the 24 kDa N-terminal DNA binding domain and the 89 kDa C-terminal catalytic domain by caspase, which is known to mediate the apoptotic process, has been used extensively as a marker of apoptosis [28]. Figure 4 shows that ischemia/reperfusion increased the level of the C-terminal 89 kDa PARP fragment, indicating increased apoptosis in these cardiomyocytes. When the heart was perfused in the presence of MTB, there was a significant reduction in the level of the PARP fragment (Fig. 4).

3.2. Effect of MTB on SAP kinase and p38 MAPK activity in the isolated heart

Activation of SAP kinase is one of the mechanisms leading to apoptosis. The effect of MTB on SAP kinase activity was first studied using the isolated rat heart as a model system. Rat hearts were perfused in the presence or absence of 1 and 10 μg/mL of MTB and subjected to ischemia or ischemia/reperfusion; then the cellular SAP kinase activity was determined. As previously demonstrated by other investigators [3,5], there was no activation of SAP kinase when the rat heart was subjected to ischemia alone (Fig. 5A). The addition of MTB produced a slight but not significant inhibition of SAP kinase activity. In contrast, SAP kinase activity was elevated significantly (by 1.7-fold) during ischemia/reperfusion. Figure 5A shows that this elevation in SAP kinase activity was transiently abolished (i.e. in the first 30 min) in the presence of 1 μg/mL of MTB. Similar results were obtained when hearts were perfused with 10 μg/mL of MTB (data not shown).

Since p38 MAPK has also been implicated in apoptosis associated with ischemia/reperfusion [3], the effect of MTB on this kinase activity was also investigated in our model system. Consistent with published reports, there was a transient activation of p38 MAPK activity during ischemia (Fig. 5B). In the presence of MTB, there was no significant decrease in the p38 MAPK activity. When rat hearts were subjected to ischemia/reperfusion, there was a significant increase in p38 MAPK activity after 30 min of ischemia. Similarly, there was no significant decrease in p38 MAPK activity in the ischemic/reperfused heart in the presence of MTB.

3.3. Direct effect of MTB on SAP kinase activity

The direct effect of MTB on SAP kinase activity was then investigated. Ischemic/reperfused heart extracts were used as the source of SAP kinase for this part of the study. Figure 6 shows that in the presence of 0.01 μg/mL of MTB, there was a 27% reduction in SAP kinase activity. Further addition of the compound up to 10 μg/mL resulted in a greater reduction of the SAP kinase activity (to 56%) compared with control activity. Addition of a higher concentration of MTB (100 μg/mL) did not result in greater inhibition of SAP kinase activity (data not shown).

Next, the mechanism for the MTB inhibition of SAP kinase was investigated. The determination of SAP kinase activity involved two separate steps. In the first step (binding step), immobilized c-Jun was used to "pull down" SAP kinase in the tissue extract, while the second step (kinase step) involved the actual phosphotransferase reaction. Rat hearts were homogenized in cell lysis buffer, and the soluble extracts were used for determination of SAP kinase activity. As depicted in Fig. 7, in the presence of MTB (1 μg/mL), the c-Jun binding activity of SAP kinase was enhanced significantly by 50%. In contrast, phosphotransferase activity of the SAP kinase was reduced to 63% of control. However, the addition of MTB resulted in an overall 44% inhibition of the SAP kinase reaction. Similar results were observed when 10 μg/mL of MTB was included in the assays for SAP kinase activity (Fig. 7). The mechanism by which MTB

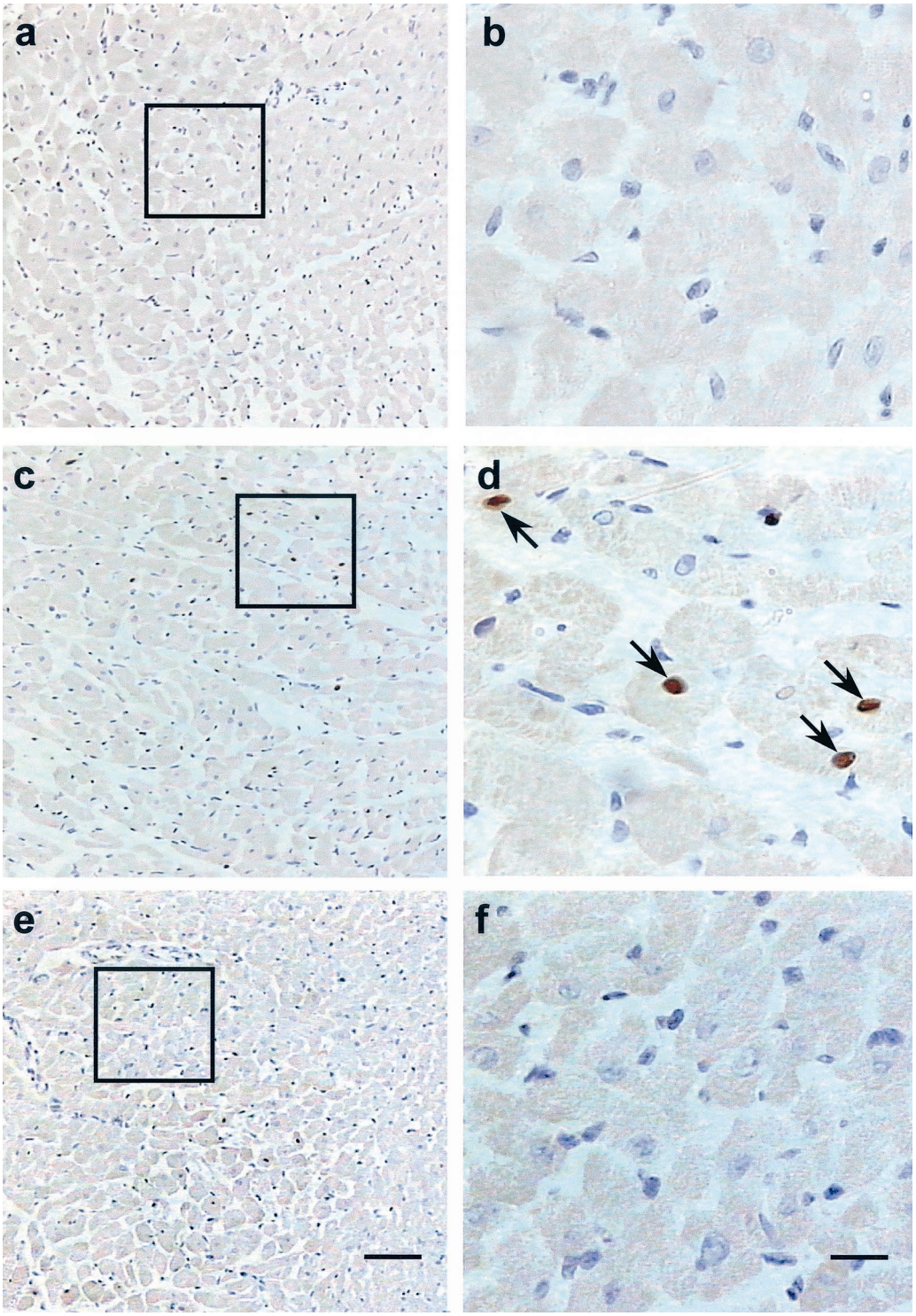

Fig. 2. TUNEL analysis of apoptosis in the ischemic/reperfused heart. Light photomicrograph of TUNEL-stained 1-μm sections of MTB-treated (panels a and b) and untreated (panels c and d) ischemic/reperfused rat heart, and non-ischemic control (panels e and f). Panels b, d, and f give a high-power view of the areas indicated in panels a, c, and e, respectively. The arrows in panel d point to TUNEL-positive nuclei. The bar for panels a, c, and e = 100 μm; the bar for panels b, d, and f = 25 μm.

altered SAP kinase activity was further examined by kinetic analysis. The data obtained from the Lineweaver–Burk plot of phosphorylated c-Jun formation versus ATP are summarized in Table 1. Compared with the uninhibited enzyme, there was a 2-fold increase in the apparent K_m with no change in the apparent V_{max} for the MTB-inhibited kinase. Additionally, the V_{max}/K_m of the inhibited enzyme was almost halved.

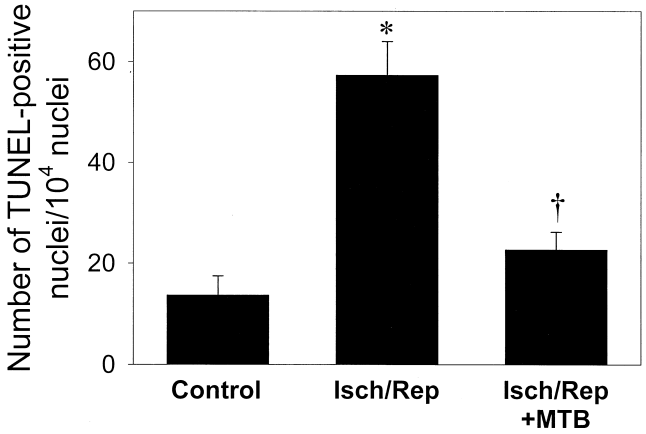

Fig. 3. Effect of MTB on apoptosis. The number of TUNEL-positive nuclei per 10,000 nuclei is shown for non-ischemic control, untreated ischemic/reperfused (Isch/Rep), and MTB-treated ischemic/reperfused hearts. Data represent means ± SD (N = 3–4 from each group). Key (*) $P < 0.001$ compared with the non-ischemic control, and (†) $P < 0.001$ compared with Isch/Rep.

3.4. Effect of MTB on nuclear localization of active SAP kinase

The subcellular localization of SAP kinase was investigated in the MTB-treated hearts. Nuclear, membrane, and cytosolic extracts from MTB-treated or untreated ischemic/reperfused hearts were analyzed for SAP kinase activity. In the ischemic/reperfused rat heart, there was a 33% increase in the specific activity of SAP kinase in the nucleus (63.1 ± 4.3 relative densitometric units) compared with the non-ischemic heart (47.4 ± 6.0 relative densitometric units). Treatment with MTB (1 μg/mL) significantly reduced the nuclear SAP kinase activity in the ischemic/reperfused heart (38.3 ± 6.2 relative densitometric units, $P < 0.01$ compared with the ischemic/reperfused heart). In contrast, there was no significant change in the specific activity of SAP kinase in the cytosolic fraction of the ischemic/reperfused heart, with or without MTB treatment. There was also a significant 46% increase in the specific activity of SAP kinase in the membrane fraction (27.1 ± 3.2 relative densitometric units) of the ischemic/reperfused heart when compared with that in the non-ischemic control (18.6 ± 2.6 relative densitometric units). As observed for the nuclear fraction, treat-

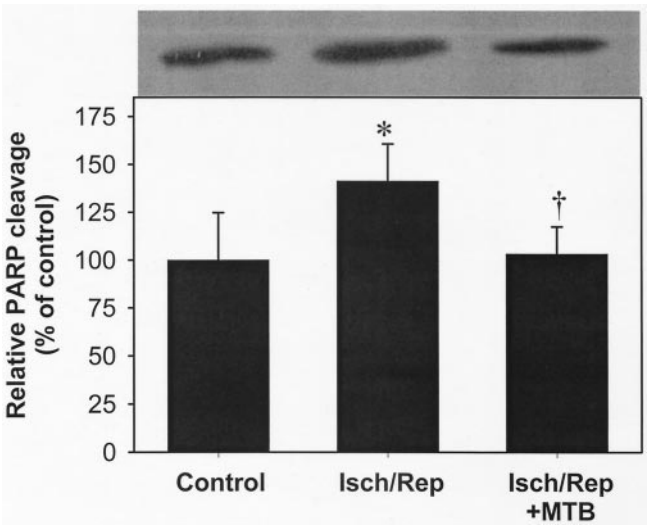

Fig. 4. Effect of MTB on PARP cleavage. Cellular extracts from ischemic/reperfused (Isch/Rep) hearts, perfused in the absence and presence of 1 μg/mL of MTB, were subjected to SDS–PAGE and western blot analysis, using a rat-specific polyclonal antibody against cleaved PARP (D214) (Cell Signaling Technology). The upper panel shows a representative western blot of the cleaved PARP fragment. Bar chart values represent means ± SEM (N = 4–5). Control value = 19.6 ± 2.2 relative densitometric units. Key: (*) $P < 0.05$ compared with the non-ischemic control, and (†) $P < 0.05$ compared with Isch/Rep.

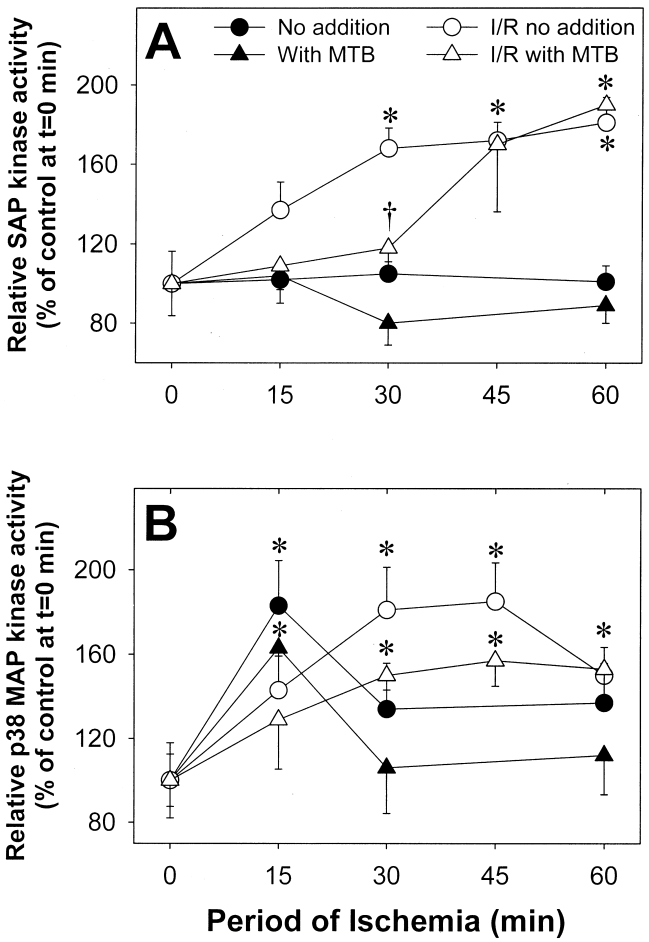

Fig. 5. Selective inhibition of SAP kinase activity by MTB in the ischemic/reperfused (I/R) heart. Isolated rat hearts, perfused in the absence (circles) or presence (triangles) of 1 μg/ml of MTB, were subjected to either 30-min global ischemia alone (filled symbols) or 30-min global ischemia followed by 20-min reperfusion (open symbols). Normal perfused heart was used as the control. (A) Relative SAP kinase activity (means ± SEM) from N = 4–6 separate experiments expressed as a percentage of the control (t = 0 min). Control = 21.2 ± 3.3 relative densitometric units. (B) Relative p38 MAPK activity (means ± SEM) from N = 4–6 separate experiments expressed as a percentage of control (t = 0 min). Control = 27.5 ± 2.4 relative densitometric units. Key: (*) $P < 0.05$ compared with the control; and (†) $P < 0.05$ compared with corresponding heart perfused in the absence of MTB.

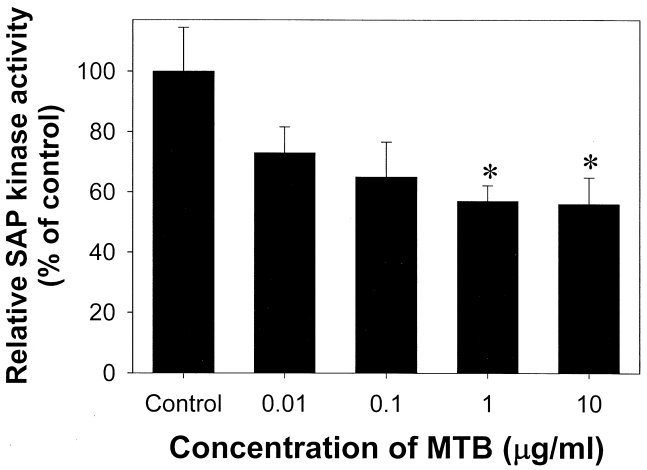

Fig. 6. *In vitro* effect of MTB on SAP kinase activity. Phosphotransferase activity of SAP kinase was determined in the absence (control, 0 μg/mL = 54.4 ± 6.6 relative densitometric units) or presence of various concentrations of MTB. Western blot and the subsequent quantitation of the phosphorylated GST-c-Jun were performed. Each value represents the mean ± SEM (N = 4–6). Key: (*) $P < 0.05$ compared with the control.

ment with MTB significantly reduced the membrane-bound SAP kinase activity in the ischemic/reperfused heart (19.6 ± 0.9 relative densitometric units).

3.5. Correlation between MTB inhibition of SAP kinase activity and apoptosis

To evaluate the relationship between the number of TUNEL-positive cells and SAP kinase, a concentration–response experiment using the isolated ischemic/reperfused rat heart was performed. In the presence of 0.01 μg/mL of MTB, the activated SAP kinase activity was inhibited by 32% (Fig. 8, top panel). There was no change in the number of TUNEL-positive cells at this MTB concentration. When the concentration of MTB was increased to 0.1 μg/mL, the percent inhibition of activated SAP kinase activity was increased to 36%. At this concentration, the number of TUNEL-positive cells was also decreased from 58 ± 6 to 47 ± 5 cells (Fig. 8, top panel). At 1 μg/mL of MTB, the activation of SAP kinase was abolished, and there was no difference in the number of TUNEL-positive cells in the ischemic/reperfused heart when compared with that of the non-ischemic control. The bottom panel of Fig. 8 shows a correlation between the number of TUNEL-positive cells and SAP kinase activity (Spearman rank correlation coefficient [R_s] = 0.721, P = 0.002).

Table 1
Apparent kinetic parameters of SAP kinase in the presence and absence of MTB

Experiment	K_m (μM)	V_{max} (fmol/min)	V_{max}/K_m (fmol/min · μM)
No MTB	10.7	2.8	0.26
MTB (1 μg/mL)	18.8	2.7	0.14

Values of the apparent K_m and V_{max} were determined from linear regression analysis of two experiments. Experimental differences were less than 10%.

4. Discussion

The present study demonstrated that MTB, a bioactive compound isolated from Danshen, protected the ischemic/reperfused heart against apoptosis. Our results suggest that this cardioprotective effect of MTB may have resulted from an attenuation of SAP kinase activity in the heart, representing one of the mechanisms contributing to the anti-apoptotic effect.

A novel finding of this study is that the administration of MTB markedly attenuated the activation of SAP kinase following myocardial ischemia/reperfusion. The inhibitory activity of MTB is through (i) a direct inhibition of the phosphotransferase activity of SAP kinase, and (ii) a reduced nuclear translocation of the activated kinase. At low micromolar concentrations, MTB was effective in inhibiting SAP kinase activity. Kinetic analysis revealed that the apparent K_m value for ATP was greater in the presence of MTB, indicating a decrease in the affinity of the kinase for ATP. In combination with an unchanged $V_{max,}$ a competitive type of inhibition by MTB is indicated. Furthermore, a decreased apparent V_{max}/K_m ratio also suggested a decreased efficiency in the utilization of ATP by SAP kinase in the presence of MTB. This competitive inhibition of SAP kinase by MTB is probably reversible. If MTB interacts irreversibly with SAP kinase, then the omission of MTB only during the second phosphotransferase step of the SAP kinase assay should still result in an inhibition of kinase activity. However, this was not the case. When MTB was present only during the incubation period for binding of SAP kinase to c-Jun, an increase in the activity of SAP kinase was detected. It is interesting that MTB at the same time also enhanced the binding of SAP kinase to c-Jun, which may result in a more rapid enzyme reaction (phosphotransferase reaction). Karin and colleagues [29] have determined the protein domain in c-Jun that was involved in the binding of SAP kinase. MTB may act by opening up this region of c-Jun to allow its binding to SAP kinase. Recently, the presence of a JNK(SAP kinase)-interacting protein (JIP) has been reported. It has been proposed that JIP may act as a scaffold protein that can link upstream kinases to SAP kinase, thus providing specificity and enhanced activation of the stress-activated kinase cascade [30]. It remains to be investigated whether MTB enhances binding of SAP kinase to c-Jun via the interaction with JIP.

Apoptosis has been suggested to account for a significant proportion of cardiomyocyte death observed in reperfusion injury [13,31]. Reperfusion is a phenomenon that normally follows ischemic episodes, which underlies myocardial infarction, thrombotic stroke, embolic vascular occlusions,

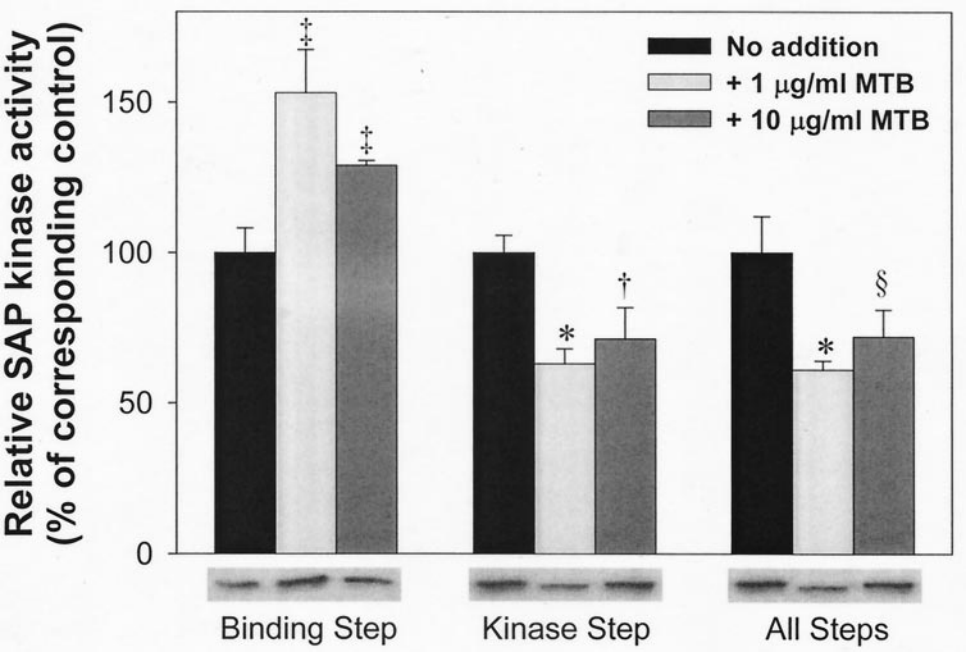

Fig. 7. Effect of MTB on c-Jun binding and phosphotransferase activities of SAP kinase. The lower panel shows representative autoradiograms of phosphorylated GST-c-Jun as a measurement of SAP kinase activity. Bar chart values (N = 5–8) represent SAP kinase activity (means ± SEM) expressed as a percentage of the corresponding control. Control value for binding was 51.0 ± 3.9 relative densitometric units; kinase step = 50.7 ± 3.0 relative densitometric units; both = 51.0 ± 7.4 relative densitometric units. Key (*) $P < 0.001$, (†) $P < 0.01$, (‡) $P < 0.02$, and (§) $P < 0.05$ compared with the corresponding control.

angina pectoris, peripheral vascular insufficiency, cardiac surgery, and organ transplantation [32]. The attenuation of reperfusion injury and cell loss is an important process in the functional recovery of the heart. Activation of SAP kinase is damaging for the heart since it may lead to apoptosis of cardiomyocytes. The decrease in the activation of SAP kinase by MTB may lead to a decrease in apoptosis, thus reducing the injurious effect of reperfusion. In this study, a positive correlation was obtained on the number of TUNEL-positive cells and the level of SAP kinase activity. Results also indicated that there was an increased level of apoptosis in the ischemic/reperfused isolated heart. This was evident from the increased number of TUNEL-positive cells and the level of PARP fragments. The presence of MTB in the perfusate significantly attenuated these cellular markers of apoptosis. At a low concentration (10 ng/mL), MTB was able to inhibit 32% of the activated SAP kinase activity while having no significant effect on apoptosis. When higher concentrations of MTB were used, a further inhibition of SAP kinase and an attenuation of apoptosis were observed. These results indicated that the complete inhibition of apoptosis might require the inhibition of several processes or signal transduction pathways. It may be the combination of these effects and that of the direct/indirect inhibition of SAP kinase that may contribute to the cardioprotective effect observed in this study.

There may be additional mechanisms for the protective effect of MTB against apoptosis. Since oxidative stress resulting from myocardial ischemia can stimulate the activation of SAP kinase [6,33], injuries caused by the oxidative stress may be alleviated by the inhibition of SAP kinase. In a separate study, we have demonstrated that MTB also possesses a strong inhibitory effect on the oxidative modification of low-density lipoprotein [34]. Therefore, the antioxidant effect [34,35] and the reported free radical scavenging activity [36] of MTB may also be responsible for the inhibitory action on apoptosis. There is also the possibility for the involvement of PKC-ϵ in the activation of SAP kinase during ischemic preconditioning [37]. However, there is also evidence that the induction of apoptosis via the SAP kinase pathway may be independent of protein kinase C [38]. Other pathways leading to apoptosis via the activation of SAP kinase are yet to be discovered. Additionally, there are other independent pathways that may lead to apoptosis. Ma and colleagues [39] recently demonstrated that inhibition of p38 MAPK could attenuate reperfusion injury causing apoptosis. However, the use of a specific inhibitor of p38 MAPK failed to completely inhibit apoptosis. They suggested that signal transduction pathways such as JNK/SAP kinase may also contribute to the apoptotic process. The crosstalk of these signaling pathways leading to apoptosis will be the subject of further study.

Data from the present study also demonstrate an inhibition of the translocation of activated SAP kinase after ischemia/reperfusion. It is interesting to note that there was a significant increase in active SAP kinase in the membrane fraction. Since the mechanism of translocation of SAP kinase from the cytosol to the nucleus is still unknown, it

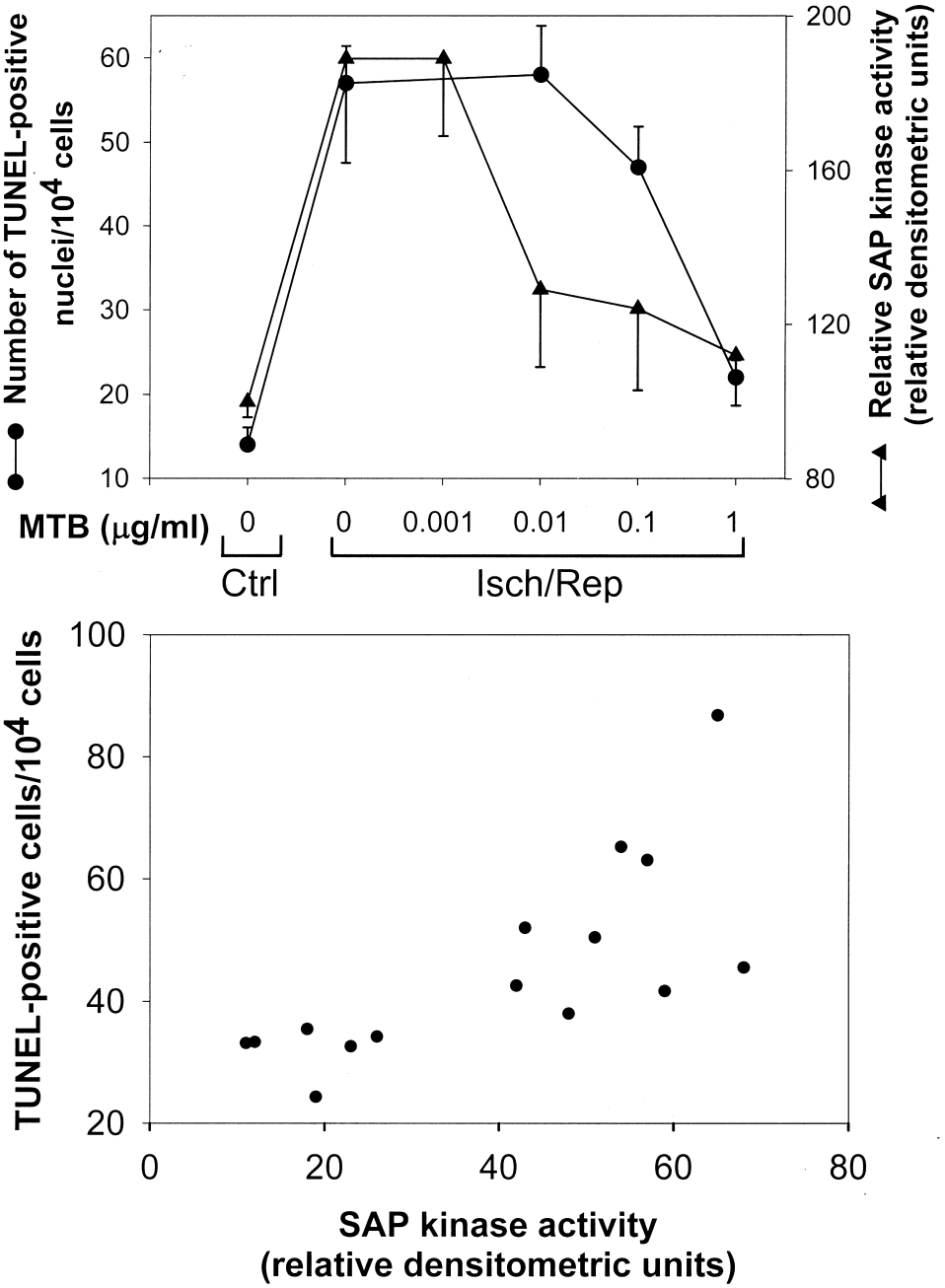

Fig. 8. Correlation between TUNEL positive cells and SAP kinase activity. (Top panel) Isolated rat hearts (N = 4–5), perfused in the absence or presence of various concentrations of MTB, were subjected to ischemia/reperfusion (Isch/Rep) followed by TUNEL staining and determination of SAP kinase activity. Relative SAP kinase activity (means ± SEM) is expressed as a percentage of the non-ischemic perfused control (Ctrl = 21.3 ± 0.9 relative densitometric units). (Bottom panel) Scatter plot diagram showing the correlation between TUNEL-positive cells and SAP kinase activity (N = 15). Spearman rank correlation analysis yielded a coefficient [R_s] = 0.721 (P = 0.002).

remains possible that the membrane-bound SAP kinase represents an intermediate in the translocation process. SAP kinase may also be associated with the membrane during its activation since MEKK, a kinase that is upstream in the pathway leading to the stimulation of SAP kinase, has been found predominantly in Golgi-associated vesicles [40]. In the nucleus, the active SAP kinase may, in turn, phosphorylate transcription factors such as c-Jun, ATF2, p67SRF, p62TCF, and Sap-1a [41–43]. Some of these transcription factors are thought to play an important role in determining the apoptotic death of the myocardial cell [44–46]. The apoptotic myocardial cell death has been observed in both human hearts [13,47] and the hearts of experimental animals [3,48,49]. Alternatively, activation of SAP kinase may be involved in the induction process of apoptosis that is initiated in the mitochondria [50].

In summary, the present study clearly demonstrates the anti-apoptotic effect of MTB, a purified compound from Danshen. This cardioprotective effect of MTB may be exerted via its direct/indirect inhibition of SAP kinase activity and the reduction in nuclear translocation of the active kinase.

Acknowledgments

The authors wish to acknowledge Mr. W.M.K. Leung for his excellent technical assistance. The work described here was supported by grants from the Research Grant Council of Hong Kong Special Administrative Region, China (HKU 7297/98M and 7356/00M), the National Science Foundation of China/Research Grant Council of HKSAR Joint Research Scheme (NSFC/HKU 39), and an Outstanding Researcher Award to Y.L.S. and a Sir Edward Youde Memorial Fellowship to K.K.W.A-Y.

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