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Antiapoptotic mechanisms of benidipine in the ischemic/reperfused heart

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1 Considerable evidence indicates that calcium plays a critical role in apoptosis. We have previously shown that benidipine, a vasodilatory calcium channel blocker, attenuates postischemia myocardial apoptosis. The present study was designed to determine the mechanisms by which benidipine exerts its antiapoptotic effect.

2 Adult male rats were subjected to 30 min of ischemia followed by 3 h of reperfusion. Rats were randomized to receive either vehicle or benidipine (10 $\mu g\,kg^{-1}$, i.v.) 10 min before reperfusion.

3 Compared with rats receiving vehicle, those rats treated with benidipine had reduced postischemic myocardial apoptosis as evidenced by decreased TUNEL-positive staining (8.4 ± 1.2 vs $15.3\pm1.3\%$, $P<0.01$) and caspase-3 activity (1.94 ± 0.25 vs 3.43 ± 0.29, $P<0.01$).

4 Benidipine treatment significantly reduced mitochondrial cytochrome *c* release and caspase-9 activation, but had no effect on caspase-8 activation, suggesting that benidipine exerts its antiapoptotic effect by inhibiting the mitochondrial-mediated, but not death receptor-mediated, apoptotic pathway.

5 Benidipine treatment not only increased the maximal activity of ERK1/2 at 10 min after reperfusion, but also prolonged the duration of ERK1/2 activation. Benidipine treatment had no significant effect on other apoptotic regulating molecules, such as p38 MAPK.

6 Taken together, our present study demonstrated for the first time the differential regulation of a calcium channel blocker. Benidipine tilted the balance between ERK1/2 and p38 MAPK toward an antiapoptotic state, decreased mitochondrial cytochrome *c* release, reduced caspase-9 activation, and attenuated subsequent caspase-3 activation and postischemic myocardial apoptosis.
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Abbreviations: AAR, area-at-risk; ANAR, area-not-at-risk; MI/R, myocardial ischemia/reperfusion

Introduction

Growing evidence from both animal experiments and clinical observations indicates that myocardial infarction after ischemia/reperfusion (MI/R), the single-most important cause of death in the United States, is caused not only by necrosis but also by apoptosis. We and others have recently demonstrated that blocking the signal transduction leading to apoptosis significantly reduces myocardial infarct size and improves cardiac function, suggesting that apoptosis contributes to myocardial reperfusion injury, and that antiapoptotic treatment may improve clinical outcomes in patients with ischemic heart disease.

It has been long recognized that ischemia, and ischemia followed by reperfusion, cause intracellular calcium overloading in cardiac cells and that administration of calcium antagonists before ischemia significantly reduces myocardial infarct size and improves myocardial functional recovery after reperfusion (Lefer *et al*., 1979). In a recent study, we have demonstrated that administration of benidipine, a long-lasting vasodilating calcium antagonist, reduced cytosolic calcium levels, decreased myocardial apoptosis, and significantly improved cardiac contractile function after ischemia and reperfusion. Administration of benidipine at a higher dose reduced cardiac work and decreased not only apoptotic death, but also necrotic cell death (Gao *et al*., 2001). These results suggest that blockade of calcium overload in ischemic-reperfused cardiomyocytes inhibits apoptotic signaling in a hemodynamic-independent fashion. However, the mechanism underlying benidipine's antiapoptotic effect in the ischemic/reperfused heart remains unknown.

Apoptosis is regulated by two principal pathways – intrinsic and extrinsic. The extrinsic pathway involves the binding of soluble or cell membrane-bound ligands to cell surface receptors such as Fas and tumor necrosis factor receptor 1 (TNFR1), and the subsequent recruiting of procaspase-8 through homotypic interactions involving death effector motifs. The close proximity of procaspase-8 stimulates its

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autoactivation, after which caspase-8 activates downstream caspases and induces apoptosis (Konopleva *et al*., 1999). The intrinsic pathway is initiated by mitochondria as a result of diverse pathologic stimuli such as nutrient and growth/survival factor deprivation, hypoxia, and oxidative stress. These pathologic stimulations result in the translocation of cytochrome *c* from the mitochondrial intermembrane space and inner membrane to the cytoplasm. Cytochrome *c* then binds to and stimulates the oligomerization of Apaf-1 in the presence of dATP and results in the subsequent recruitment and activation of procaspase-9. This leads to the activation of downstream procaspase-3, -6, and -7, proteolysis of specific cellular substrates and cell death (Brenner & Kroemer, 2000). Whether benidipine inhibits the intrinsic, extrinsic, or both pathways, thus reducing postischemic myocardial apoptosis, has never been previously studied.

Therefore, the aims of the present study were (1) to determine the apoptotic pathways (i.e., death receptor pathway or mitochondrial pathway) that can be blocked by benidipine in the ischemic/reperfused heart; and (2) to investigate whether benidipine may reduce proapoptotic activity and/or enhance antiapoptotic activity in the ischemic/reperfused heart, thus tilting the anti- and proapoptotic balance toward a prosurvival state and reducing postischemic apoptosis.

Methods

Materials

Benidipine, a long-acting 1,4-dihydropyridine calcium antagonist (Kitakaze *et al*., 1999), was provided by Kyowa Hakko Kogyo Co., LTD (Tokyo, Japan). All other compounds were purchased from Sigma Chemicals Co. (St Louis, MO, U.S.A.). The experiments were performed in adherence to NIH Guidelines on the Use of Laboratory Animals and were approved by the Thomas Jefferson University Committee on Animal Care.

Experimental preparation

Male Sprague–Dawley rats were anesthetized with 2% isoflurane. Myocardial ischemia was produced by temporarily exteriorizing the heart (<1 min) *via* a left thoracic incision and placing a 6-0 silk suture slipknot around the left anterior descending coronary artery. After 30 min of ischemia, the slipknot was released and the myocardium was reperfused for 3 h. At 10 min before reperfusion, rats were randomized to receive vehicle (0.15% Tween 80 in saline) or benidipine ($10\,\mu g\,kg^{-1}$). This dose was chosen based on our previous study showing that administration of benidipine at this dose significantly reduces calcium overload in ischemia/reperfused cardiac tissue and decreases postischemic myocardial apoptosis (Gao *et al*., 2001). Drug or vehicle was given intravenously over 1 min. Sham-operated control rats (Sham MI/R) underwent the same surgical procedures except that the suture that was passed under the left coronary artery was not tied. At the end of the 3-h reperfusion period, the ligature around the coronary artery was retied and 1 ml of 2% Evans blue dye was injected into the left ventricular cavity. The dye was circulated and uniformly distributed except in that portion of the heart previously perfused by the occluded coronary artery (area-at-risk, AAR). The heart was quickly excised, the AAR was isolated and cardiac tissue was processed according to the procedures described below for immunohistological ($n = 5$–6/group), biochemical ($n = 10$–12/group) and Western blot assays ($n = 5$–6/group).

Determination of myocardial apoptotic death

Myocardial apoptosis was determined by TUNEL staining and caspase activity assay as described previously (Gao *et al*., 2002). In brief, at the end of experiment, the hearts were perfused first with 0.9% NaCl for 5 min and then with 4% paraformaldehyde in PBS (pH 7.4) for 20 min. Four longitudinal sections from ischemic regions were cut and further fixed in 4% paraformaldehyde in PBS for 24 h at room temperature. Fixed tissues were embedded in a paraffin block and two slides at 4–5 μm thickness were cut from each tissue block. Immunohistochemical procedures for detecting apoptotic cardiomyocytes were performed by using an apoptosis detection kit (Boehringer Mannheim, Ridgefield, CT, U.S.A.) according to the manufacturer's instructions. An additional staining was performed with monoclonal anti-α-sarcomeric actin. This staining enables the identification of myocytes and, therefore, a distinction between myocyte nuclei and nuclei of other cells in the cardiac tissue. After rinsing with PBS, slides were coverslipped with mounting medium containing DAPI to permit total nuclei counting.

For each slide, 10 fields were randomly chosen, and using a defined rectangular field area ($\times 20$ objective), a total of 100 cells per field were counted. The index of apoptosis was determined (i.e., number of positively stained apoptotic myocytes/total number of myocytes counted $\times 100$) from a total of 80 fields per heart (counted as $n = 1$, at least five hearts were studied in each group). Assays were performed in a blinded manner.

Cardiac caspase(-3, -8, and -9) activity was performed by using caspase colorimetric assay kits (Chemicon International, Inc., Temecula, CA, U.S.A.) following the manufacturer's instructions. In brief, myocardial tissue was homogenized in ice-cold lysis buffer for 30 s using a PRO 200 homogenizer. The homogenates were centrifuged for 5 min at $10{,}000 \times g$ at 4°C, supernatants were collected, and protein concentrations were measured by the bicinchoninic acid method (Pierce Chemical, Rockford, IL, U.S.A.). To each well of a 96-well plate, supernatant containing 200 μg of protein was loaded and incubated with 25 μg Ac-DEVD-*p*NA, Ac-IETC-*p*NA or Ac-LEHD-*p*NA at 37°C for 1.5 h. *p*NA was cleaved from DEVD (by caspase-3), IETC (by caspase-8) or LEHD (by caspase-9) and the free pNA was quantified using a SpectraMax-Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.) at 405 nm. Changes in caspase activity in ischemic/reperfused tissue samples were calculated against the mean value of caspase activity in sham MI/R tissue and expressed as fold increase over sham MI/R.

Quantification of cytochrome c *release*

Mitochondrial cytochrome *c* release was determined as described by Ott *et al*. (2002) with modifications. In brief, 25 mg of myocardial tissue was minced on ice, resuspended in 500 μl of MSH buffer (210 mM mannitol/70 mM sucrose/5 mM HEPES, pH 7.5) supplemented with 1 mM EDTA, and homogenized with a glass Dounce homogenizer and Teflon

pestle. Cytosolic and mitochondrial fractions were separated by differential centrifugation (5 min at $1000 \times g$, 30 min at $17{,}530 \times g$). The mitochondrial pellet was resuspended in MSH buffer, sonicated with Dismembrator for 20 s on ice, and centrifuged at $17{,}530 \times$ g for 30 min at 4°C. The resulting supernatant containing mitochondrial extract from this last centrifuge or cytosolic extract from the first centrifugation (5 min at 1000 g) were separately mixed with Laemmli's loading buffer, boiled for 5 min, and subjected to SDS/10–15% PAGE at 100 V followed by electroblotting to nitrocellulose membranes for 2 h at 10 V. Membranes were blocked for 1 h with 5% nonfat milk in PBS at room temperature and subsequently probed overnight with an anti-cytochrome *c* antibody. The membranes were rinsed and incubated with a horseradish-peroxidase-conjugated secondary antibody. After secondary antibody incubation, the membranes were rinsed, and bound antibodies were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical) according to the manufacturer's instructions. The membrane was scanned with a Kodak Image Station 400 and individual band density was analyzed with Kodak 1D software. Results were expressed as cytosolic/mitochondrial cytochrome $c \times 100\%$.

Detection of ERK1/2 phosphorylation

Heart tissue samples were lysed with lysis buffer. After sonication, the lysates were centrifuged, proteins were separated by electrophoresis on SDS–PAGE and transferred onto a polyvinylidene difluoride (PVDF)-plus membrane. After being blocked with 5% milk, the immunoblots were probed with anti-pERK1/2 antibody overnight at 4°C followed by incubation with secondary antibody at room temperature for 1 h. The blots were detected using a Super Signal Western Pico Chemiluminescent Substrate (Pierce Chemical) according to the manufacturer's instructions and visualized with a Kodak Image Station 400. The blot densities were analyzed with Kodak 1D software.

Measurement of ERK1/2 and p38 MAPK activity

The ERK1/2 and p38 MAP kinase activity assays were performed by using ERK1/2 or p38 MAPK assay kits (Cell Signaling Technology) according to the manufacturer's instructions. In brief, ischemic/reperfused (30 min of ischemia followed by different periods of reperfusion as indicated in the results) heart tissue (20–25 mg) was homogenized in 0.5 ml ice-cold cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mg ml^{-1} leupeptin, 1 mM PMSF). The lysates were then sonicated on ice and centrifuged at $15{,}000 \times g$ for 10 min at 4°C. Immunoprecipitation was performed by adding 20 μl of resuspended immobilized monoclonal antibody against phospho-ERK1/2 (Thr202/Tyr204) or phospho-p38 MAP kinase (Thr180/Tyr182) to 200 μl tissue lysate containing 500 μg protein. The mixture was incubated with gentle rocking overnight at 4°C. After centrifuging at $10{,}000 \times g$ at 4°C for 2 min, the pellets were washed twice with lysis buffer and twice with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mN $MgCl_2$). The kinase reactions were carried out in the presence of 200 μM ATP and 2 μg EIk-1 fusion protein (for ERK1/2) or ATF-2 fusion protein (for p38 MAPK) at 30°C for 30 min. After incubation, the samples were separated by SDS–PAGE, and EIk-1 or ATF-2 phosphorylation was measured by Western immunoblotting using monoclonal antibody against phosphorylated EIk-1 or ATF-2 followed by an enhanced chemiluminescent detection as described above.

Statistical analysis

Time and group differences were determined by two-way analysis of variance for repeated measures, correcting for multiple comparisons by Holm's multiple rejective method as appropriate for an overall probability level of 0.05. Nonrepetitive data were subjected to analysis of variance (ANOVA) followed by the Scheffe's correction for *posthoc t*-test comparison.

Results

Effect of benidipine on myocardial apoptosis

Consistent with the results previously reported by other investigators and our group, 30 min of myocardial ischemia followed by 3 h of reperfusion resulted in significant myocardial apoptotic death manifested by a 15-fold increase in TUNEL-positive cell labeling, strong intracellular immunostaining of cleaved caspase-3, and a 3.4-fold increase in caspase-3 activity (Figures 1 and 2). Administration of benidipine at 10 μg kg^{-1}, a dose that caused only a moderate (maximal reduction: 9.4 ± 1.6 mmHg) and transient (< 5 min) reduction in aortic blood pressure, significantly decreased myocardial apoptosis as evidenced by reduction in TUNEL-positive cells (8.4 ± 1.2 vs 15.3 ± 1.3%, $P < 0.01$, Figure 1), blockade of caspase-3 cleavage in ischemic/reperfused cardiomyocytes (Figure 2a), and decrease in caspase-3 activity (Figure 2b). Taken together, these results indicate that benidipine, an L-type calcium channel blocker, inhibited MI/R-induced myocardial apoptosis without inducing a sustained hemodynamic effect. This is consistent with a recent study demonstrating that administration of benidipine at a dose that exerted no

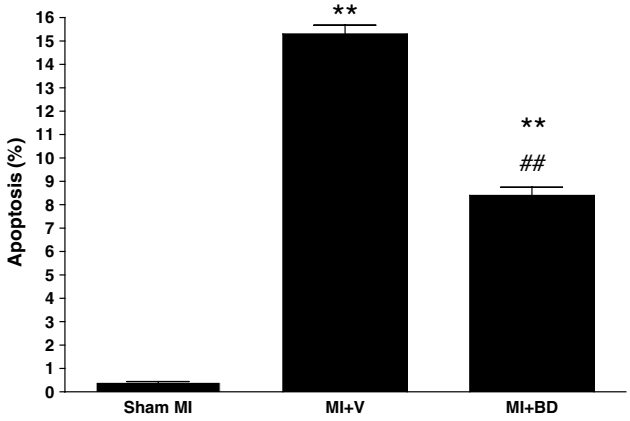

Figure 1 Effect of benidipine on postischemic myocardial apoptosis determined by TUNEL staining. Rats were subjected to 30 min of ischemia/3 h of reperfusion and treated with vehicle or benidipine. $^{**}P < 0.01$ vs sham MI/R, $^{\#\#}P < 0.01$ vs MI/R + vehicle. V = vehicle; BD = benidipine. $N = 5$–6 animals/group.

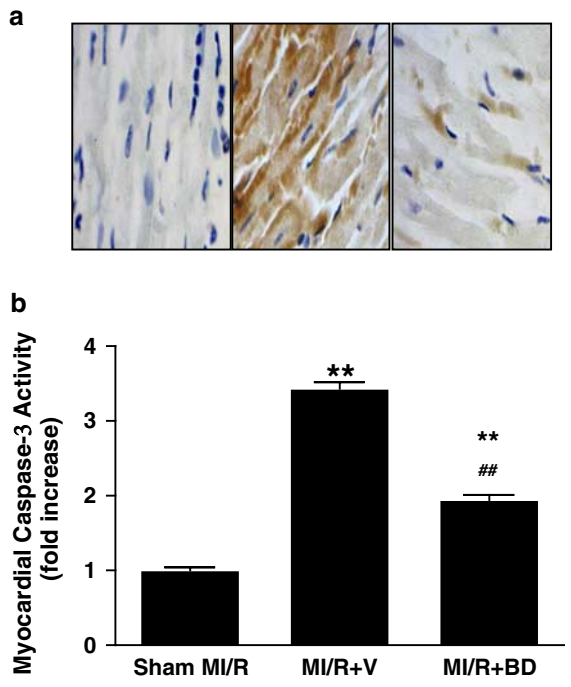

Figure 2 Effect of benidipine on caspase-3 activation in ischemic reperfused myocardial tissue. (a) Cleaved caspase-3 was immunohistologically detected in myocardial tissue by using an antibody that selectively recognizes activated caspase-3 (representative). (b) Myocardial caspase-3 activity was determined by a colorimetric kit using Ac-DEVD-*p*NA as a substrate ($N=10$–12 animals/group). $^{**}P<0.01$ vs sham MI/R, $^{\#\#}P<0.01$ vs MI/R + vehicle. V = vehicle; BD = benidipine.

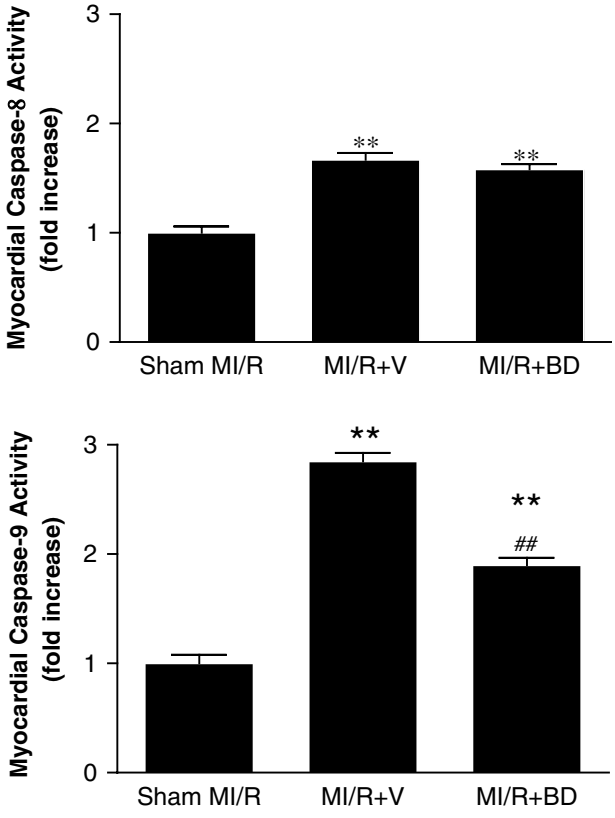

Figure 3 Effect of benidipine on caspase-8 and -9 activation in ischemic reperfused myocardial tissue. Myocardial caspase-8 and -9 activity were determined by a colorimetric kit using Ac-IETC-*p*NA (for caspase-8) or Ac-LEHD-*p*NA (for caspase-9) as a substrate ($N=10$–12 animals/group). $^{**}P<0.01$ vs sham MI/R, $^{\#\#}P<0.01$ vs MI/R + vehicle. V = vehicle; BD = benidipine.

significant hemodynamic effect markedly reduced the severity of experimental autoimmune myocarditis (Yuan *et al*., 2003).

Effect of benidipine on caspase-8 and -9 activation

Having demonstrated that benidipine reduced apoptosis and blocked the activation of caspase-3, the final common pathway in caspase-dependent apoptotic cell death, we further determined the upstream pathway(s) *via* which benidipine inhibits caspase-3 activation. At 30 min of myocardial ischemia followed by 3 h of reperfusion caused a moderate increase in caspase-8 activation (1.7-fold over sham MI/R) that was not inhibited by benidipine. In contrast, administration of benidipine markedly reduced caspase-9 activation (1.9 ± 0.24 vs 2.85 ± 0.26 in vehicle group, $P<0.01$) (Figure 3). These results demonstrated that benidipine inhibited postischemic myocardial apoptosis by blocking the intrinsic (i.e., mitochondrial-mediated), not extrinsic (i.e., death receptor-mediated), apoptosis pathway.

Effect of benidipine on mitochondrial cytochrome c *release*

To obtain further evidence that benidipine inhibits mitochondrial-mediated apoptosis, we investigated mitochondrial cytochrome *c* release, an upstream signal that activates caspase-9. As illustrated in Figure 4, in sham MI/R hearts, cytochrome *c* remained in the mitochondrial fraction, and the ratio between cytosolic cytochrome *c* and mitochondrial cytochrome *c* was less than 3%. Myocardial ischemia and reperfusion resulted in a marked increase in cytosolic cytochrome *c*, and the ratio of cytosolic/mitochondrial cytochrome *c* increased to $17\pm3.8\%$. Most interestingly, treatment with benidipine markedly reduced mitochondrial cytochrome *c* release.

Effect of benidipine on ERK1/2 and p38 MAPK activation

Our results described above clearly demonstrated that treatment with benidipine reduced mitochondrial cytochrome *c* release and subsequent caspase-9 activation, thus inhibiting postischemic myocardial apoptosis. Previous studies have demonstrated that mitochondrial permeability is controlled by the balance between antiapoptotic and proapoptotic Bcl-2 family members, which themselves are regulated by the balance of upstream regulators, such as extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt (antiapoptotic), p38 mitogen-activated protein kinase (MAPK) and JNK (c-Jun N-terminal Kinase) (proapoptotic). To further elucidate the mechanisms by which benidipine regulates mitochondrial permeability, we investigated the effect of benidipine on activation of several anti- and proapoptotic molecules that control the balance between the anti- and proapoptotic Bcl-2 family. Interestingly, treatment with benidipine significantly increased

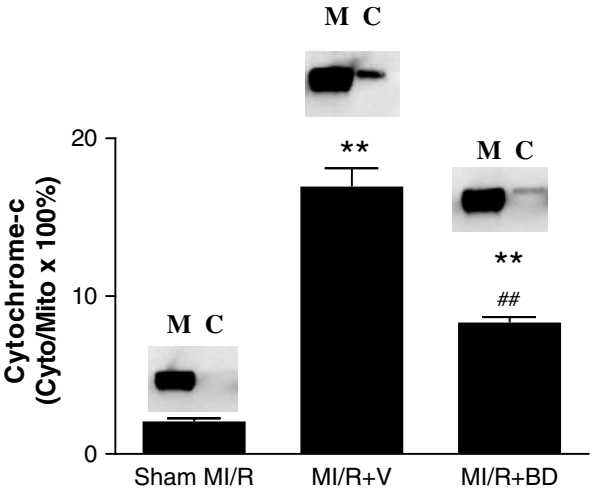

Figure 4 Effect of benidipine on mitochondrial cytochrome *c* release in ischemic reperfused myocardial tissue. Cytosolic and mitochondrial cytochrome *c* was detected separately by Western blot (representative blot on top of each bar) and their density ratio was calculated (bar heights represent mean density of 5–6 animals/group). $^{**}P<0.01$ vs sham MI/R, $^{\#\#}P<0.01$ vs MI/R + vehicle. V = vehicle; BD = benidipine. M = mitochondrial, C = cytosolic.

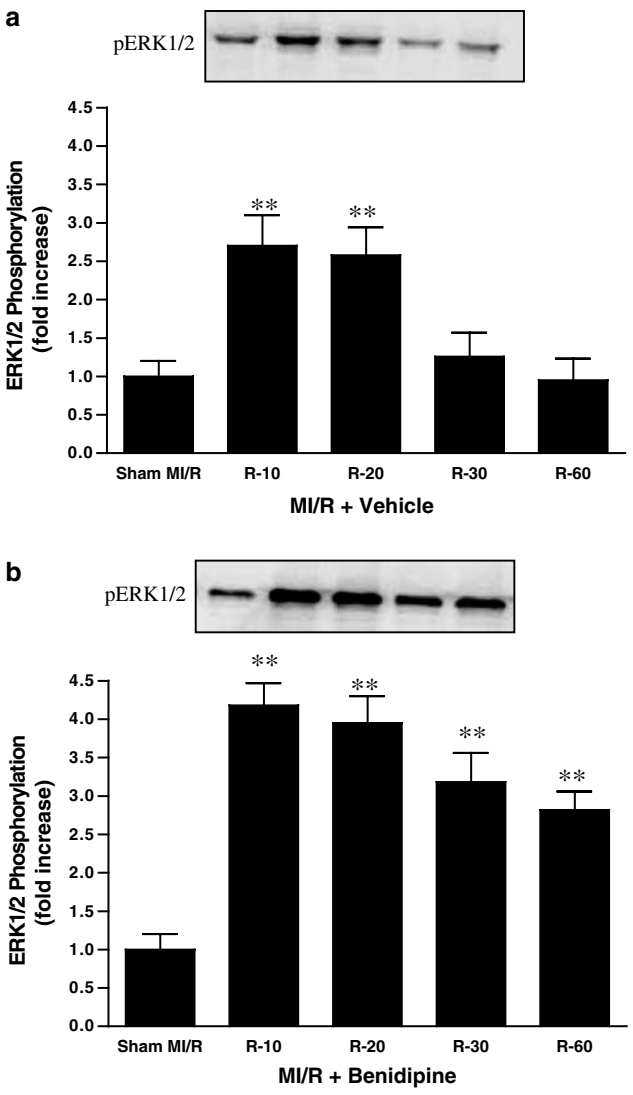

Figure 5 Effect of benidipine on ERK1/2 phosphorylation in ischemic-reperfused myocardial tissue. Phosphorylated ERK1/2 was detected with Western blot (representative blot on top of each bar) analysis by using an antibody that selectively recognizes the phosphorylated form of ERK1/2 (bar heights represent mean density of 5–6 animals/group). R10, R20, R30 and R60 = 10, 20, 30 or 60 min of reperfusion.

phosphorylation ERK1/2, an antiapoptotic molecule that reduces mitochondrial permeability, during the entire reperfusion period (Figure 5). To further assure that benidipine treatment not only increased ERK1/2 phosphorylation but also increased its activity, ERK1/2 activity was directly determined with an ELISA kit. As illustrated in Figure 6a, treatment with benidipine not only increased the maximal altitude of ERK1/2 activation occurring at 10 min after reperfusion, but also prolonged the duration of ERK1/2 activation (Figure 5). However, treatment with benidipine had no effect on activation of Akt, JNK (data not shown) and p38 MAPK (Figure 6b). These results demonstrated that benidipine treatment restored a beneficial anti-/proapoptotic MAPK balance and thus blocked mitochondrial-initiated apoptosis.

Discussion

We have made several novel observations in the present study. First, it is well described that apoptosis is initiated by either the death receptor pathway or the mitochondrial pathway in cultured cells. However, the relative importance of each pathway is likely cell type- and stimuli-dependent. We have demonstrated that in this rat model of 30 min of myocardial ischemia and 3 h of reperfusion *in vivo*, apoptosis is mediated primarily by mitochondrial cytochrome *c* release and subsequent caspase-9 activation. Second, we have provided direct evidence that treatment with benidipine, a long-lasting calcium channel blocker that is 30–100 times more potent than other dihydropyridine calcium antagonists (such as amlodipine and nifedipine) in blocking cardiomyocyte calcium channel (Yamamoto *et al*., 1990; Inomata & Tanaka, 2003), reduced postischemic myocardial apoptosis by inhibiting mitochondrial cytochrome *c* release, caspase-9 activation and subsequent caspase-3 activation. Third, treatment with benidipine not only increased the maximal activity of ERK1/2 at 10 min after reperfusion, but also slowed the decline thereafter of ERK1/2, thus maintaining ERK1/2 activity at a higher level for a longer period.

Accumulating evidence indicates that apoptosis, a special form of cell death that differs from necrosis in many aspects, plays an essential role in cardiomyocyte death after ischemia and reperfusion (Kajstura *et al*., 1996; Gottlieb & Engler, 1999). Given that apoptosis is an active, gene-directed process, inhibition of apoptosis could be achieved more successfully than prevention of necrosis, a passive form of cell death that is inflicted by an acute stimulus. One of the most widely recognized biochemical features of apoptosis is the activation of a class of cysteine proteases known as caspases (Thornberry & Lazebnik, 1998). Cells possess multiple caspases, which may work in a cascade fashion. The redundancy may serve to amplify and accelerate the response, as well as to provide multiple mechanisms for success. Two pathways have been

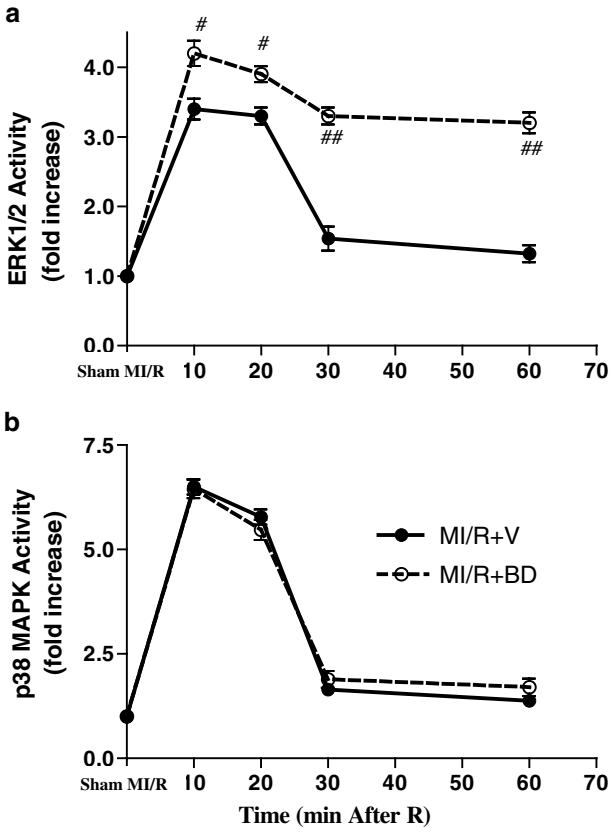

Figure 6 Effect of benidipine on ERK1/2 and p38 MAPK activity in ischemic-reperfused myocardial tissue. The ERK1/2 and p38 MAP kinase activity assays were performed by using ERK1/2 or p38 MAPK assay kits. Elk-1 fusion protein or ATF-2 fusion protein was used as substrate for ERK1/2 or p38 MAPK, respectively. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs vehicle group. $N=5$–6 animals/group. R = reperfusion.

identified that activate caspases: one activated through a cell surface signal leading to caspase-8 (and/or 10 in some cell types) activation, and another more complicated pathway involving the mitochondria and resulting in caspase-9 activation.

A variety of cellular stresses have been found to cause the disruption and collapse of the inner mitochondrial transmembrane potential, thus opening mitochondrial pores (termed 'mitochondrial permeability transition' or MPT) and resulting in the release of cytochrome *c* (Green & Reed, 1998; Yang & Cortopassi, 1998; Brenner & Kroemer, 2000). Cytochrome *c* released from the mitochondria activates caspase-9 in the presence of Apaf-1 (apoptosis protease activating factor-1) and ATP (Yang *et al*., 1997). In some cell types, activation of caspase-8 (and/or caspase-2) by the cell surface death receptor also increases mitochondrial cytochrome *c* release and subsequent caspase-9 activation by facilitating the translocation of full-length or truncated Bid (p15 Bid) from the cytosol to the mitochondrial membrane (Tafani *et al*., 2002). Several recent studies have demonstrated that in addition to this well-documented cytochrome *c*-dependent pathway, caspase-9 can also be activated by caspase-12 released from the endoplasmic reticulum (ER) under stress (Morishima *et al*., 2002). The activation of caspase-8 and -9 (initiator) followed by caspase-3 (executor) (6, and 7 in some cell types) with subsequent degradation of a variety of proteins results in irreversible damage of the cells (apoptotic cell death). Recent studies have demonstrated that formation of MPT also results in mitochondrial release of AIF (apoptosis-inducing factor), a molecule that directly results in apoptosis (Lorenzo *et al*., 1999; Arnoult *et al*., 2002).

Although apoptosis has been observed in cultured cardiomyocytes, in isolated perfused hearts, and in *in vivo* animal models as well as in clinical observations after ischemia/reperfusion (Krijnen *et al*., 2002), the signaling transduction pathway *via* which ischemia/reperfusion results in apoptosis is not well defined. Specifically, whether ischemia/reperfusion initiates myocyte apoptosis by the death receptor–caspase-8 pathway or by the mitochondria–caspase-9 pathway remains largely unknown. Our present experimental results demonstrated that ischemia/reperfusion resulted in both caspase-8 and -9 activation. However, the degree of their activation is significantly different, the increase in caspase-9 being approximately twice that of caspase-8 activation. Moreover, treatment with benidipine had no effect on caspase-8 activation but reduced caspase-9 and postischemic myocardial apoptosis to a comparable degree ($\approx 50\%$). Taken together, these results suggest that the mitochondria-initiated apoptotic pathway plays a major role in postischemic myocardial apoptosis. However, the contribution of caspase-8 to postischemic myocardial apoptosis could not be excluded from the present experimental results. In our previous study performed on rabbits (Gao *et al*., 2001), it has been demonstrated that although increasing doses of benidipine (3 times higher) further decreased infarct size (likely by a combination of antiapoptotic and antinecrotic effect), there is no significant difference in their antiapoptotic component between low- and high-dose benidipine. This result suggests that calcium overload is a significant, but not exclusive, contributor for postischemic caspase activation and subsequent apoptosis.

Under physiologic conditions, apoptosis is regulated by the balance of a variety of pro- and antiapoptotic molecules. The formation of MPT and the subsequent release of cytochrome *c* and AIF are controlled by the balance between antiapoptotic (e.g., Bcl-2, Bcl-xl, Bcl-w, Bag-1 and BI-1) and proapoptotic (e.g., Bax, Bak, Bad, Bid and Bim) Bcl-2 family members (Gross *et al*., 1999), which themselves are regulated by the balance of upstream antiapoptotic (e.g., ERK1/2 and Akt) and proapoptotic (e.g., p38 MAPK and JNK) regulators at both transcriptional and post-transcriptional levels (Buckley *et al*., 1999; Scheid *et al*., 1999). Previous studies by other investigators as well as our group have demonstrated that both ERK1/2 and p38 MAPK, two of the three members of the MAPK superfamily, are activated following myocardial ischemia and reperfusion (Clerk *et al*., 1998; Feuerstein & Young, 2000). However, activation of ERK1/2 (antiapoptotic) and p38 MAPK (proapoptotic) exert opposite effects on postischemic myocardial apoptosis (Ma *et al*., 1999; Yue *et al*., 2000). Our results demonstrated for the first time that treatment with a calcium channel blocker significantly enhanced ERK1/2 activation but had no effect on p38 MAPK activation, suggesting a differential regulation by the calcium channel blocker. Specifically in this study, benidipine tilted the balance between ERK1/2 and p38 MPAK toward an antiapoptotic state and thus have may contributed to the antiapoptotic effect observed.

Recent studies have demonstrated that calcium plays a key regulatory role in multiple sites of the signal transduction pathway leading to apoptosis in diverse cell types (McConkey & Orrenius, 1997). Calcium overloading results in overactivation

of either those enzymes that directly cause DNA fragmentation, such as calcium- and magnesium-dependent endonuclease (DNase I), or proteins that regulate signal transduction leading to apoptosis, such as calcineurin, calpain and nuclear scaffold protease (Peitsch *et al*., 1993; McConkey & Orrenius, 1997; Gottlieb & Engler, 1999). Calcium overloading also increases free radical generation (Bagchi *et al*., 1997), a well-defined apoptosis-inducing factor (Das *et al*., 1999). Moreover, in cell culture systems, addition of dihydropyridine calcium channel blockers, amlodipine and nifedipine, reduces excessive apoptosis in aging cerebellar granule cells (Mason *et al*., 1999) and in hypoxic neonatal rat cardiac myocytes (Chen *et al*., 1998). In animals subjected to renal ischemia, administration of verapamil, an L-type calcium blocker, has been demonstrated to reduce tissue apoptosis (Raafat *et al*., 1997). Taken together, these results strongly suggest that treatment with benidipine may attenuate the calcium overloading occurring in ischemic/reperfused cardiomyocytes thus reducing postischemic myocardial apoptosis (Figure 7).

In summary, we have demonstrated that administration of a calcium channel blocker before reperfusion increased ERK1/2 activity (a molecule that has been previously been shown to result in phosphorylation and inactivation of proapoptotic Bcl-2 members), decreased mitochondrial cytochrome *c* release, reduced caspase-9 activation, and attenuated subsequent caspase-3 activation and postischemic myocardial apoptosis. These results further support the notion that calcium plays a critical role in postischemic myocardial apoptosis and provide an additional mechanism by which calcium channel blockers may exert their cardioprotection against myocardial ischemia/reperfusion injury.

Limitations

Since neither a compound that directly inhibits ERK1/2 activity nor an animal model that shows selective ERK1/2 deficiency are currently available, the relation between ERK1/2 activation and reduced apoptosis in benidipine-treated animals remains correlative, rather than causative.

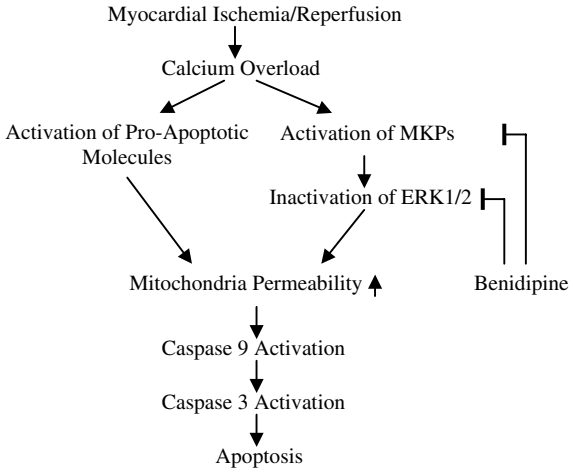

Figure 7 Schematic diagram illustrating the potential role of calcium overloading in postischemic myocardial apoptosis and proposed mechanism by which benidipine exerts its antiapoptotic effect.

In our most recent experiment, we pretreated animals with PD98059, a compound that reduces ERK1/2 activity by selective inhibition of MEK1/2 (an upstream molecule for ERK1/2 activation), and attempted to determine the role of ERK1/2 activation in benidipine's antiapoptotic effect. As expected, treatment with PD98059 alone markedly reduced ERK1/2 activation after ischemia and reperfusion. However, treatment with benidipine in PD98059 pretreated animals still significantly increased ERK1/2 activity and decreased myocardial apoptosis when compared with the group treated with PD98059 alone. This preliminary result strongly suggests that benidipine increases ERK1/2 activity by inhibiting MAPK phosphatases, such as PP2A and MKP3, that selectively inactivate ERK1/2 (Haneda *et al*., 1999; Zhou *et al*., 2002). Further study to directly address this possibility is planned.

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