Substance P induces cardioprotection in ischemia-reperfusion via activation of AKT

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1Cell Biology and Anatomy, School of Medicine, University of South Carolina, Columbia, South Carolina; 2Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin; 3Cardiovascular Center, Medical College of Wisconsin, Milwaukee, Wisconsin; and 4Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin

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Jubair S, Li J, Dehlin HM, Manteufel EJ, Goldspink PH, Levick SP, Janicki JS. Substance P induces cardioprotection in ischemia-reperfusion via activation of AKT. Am J Physiol Heart Circ Physiol 309: H676–H684, 2015. First published June 10, 2015; doi:10.1152/ajpheart.00200.2015.—Accumulating evidence indicates that substance P is cardioprotective following ischemia-reperfusion primarily due to its potent coronary vasodilator actions. However, an anti-apoptotic effect of substance P has been observed in tenocytes following ischemia, which involved activation of the AKT pathway. This suggests the possibility that substance P also provides cardioprotection via direct actions to activate AKT in myocardial cells. The purpose of this study was to test the hypothesis that substance P attenuates ischemia-related cell death via direct effects on myocardial cells by activating cell survival pathways. Seven-week-old male Sprague-Dawley rats, anesthetized with intraperitoneal pentobarbital sodium (100 mg/kg), were used. The ability of substance P to prevent cellular damage was assessed following ischemia-reperfusion in an isolated heart preparation and in short-term hypoxia without reperfusion using a left ventricular tissue slice culture preparation. In addition, the NK-1 receptor and AKT involvement was assessed using the NK-1 receptor antagonist L732138 and the AKT inhibitor LY294002. The results indicate that substance P reduced the ischemia-related release of lactate dehydrogenase in both preparations and the degree of apoptosis and necrosis in the hypoxic left ventricular slices, indicating its ability to attenuate cell damage; and induced AKT phosphorylation, with both the AKT inhibitor and NK-1 receptor antagonist preventing the increased phosphorylation of AKT and the ability of substance P to attenuate hypoxic cellular damage. It is concluded that substance P reduces ischemia/hypoxia-induced myocardial cell death by acting directly on cardiac cells to initiate cell survival pathways via the NK-1 receptor and AKT.

Paradoxical cardiomyocyte death and dysfunction, a phenomenon termed ischemia-reperfusion (I/R) injury. The tachykinin, substance P (SP), is located primarily in sensory nerves, which in the ventricles of the heart are commonly found surrounding coronary vessels (3); SP is also found in a small percentage of coronary endothelial cells (16). This makes SP ideally located to be released under conditions of altered coronary flow or pressure, such as occurs in ischemia. SP exerts its effects primarily through the neurokinin-1 receptor (NK-1R). Accumulating evidence indicates that SP is cardioprotective following I/R, primarily due to its potent coronary vasodilator actions, which results in improved reperfusion (19, 21, 23, 27). However, an anti-apoptotic effect of SP has been observed in tenocytes following ischemia, which involved activation of the AKT pathway (2). This latter observation suggests the possibility that SP may also provide cardioprotection via its direct actions on myocardial cells, in addition to, or instead of, its coronary vasodilatory effect. However, this remains to be determined. Therefore, the purpose of this study was to test the hypothesis that, in addition to its ability to act as a potent coronary vasodilator, SP attenuates ischemia-related cell death via direct effects on myocardial cells to initiate activation of cell survival pathways. To this end, the protective effect of SP was tested following I/R in an isolated rat heart preparation and in short-term hypoxia without reoxygenation using a left ventricular (LV) tissue slice culture preparation. The results indicate that although SP does improve reperfusion following I/R, it also induces AKT activation and protection from cell death independent of reperfusion.

MATERIALS AND METHODS

Animals. The protocols for the isolated heart experiments performed at the Medical College of Wisconsin (protocol No. 00002626) and the tissue slice experiments performed at the University of South Carolina (protocol No. 1765-100472) were approved by their respective Institutional Animal Care and Use Committees and conformed to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For both sets of experiments, 7-wk-old male Sprague-Dawley rats, purchased from Harlan Laboratories, were used. The animals were housed under standard environmental conditions and maintained on a normal rodent diet and tap water ad libitum.

Isolated heart I/R experiments. For the whole heart I/R experiments, a nonrecirculating Langendorff heart preparation was used. Rats were anesthetized with an intraperitoneal pentobarbital sodium injection (100 mg/kg). The right femoral vein was then exposed and heparin (1,000 IU) was administered intravenously. After time was allowed for the heparin to circulate, the heart was rapidly excised, the aorta cannulated, and retrograde perfusion with filtered Krebs-
Henseleit buffer containing (in mM) 119.1 NaCl, 4.75 KCl, 1.19 MgSO₄, 1.19 K₂HPO₄, 25.0 NaHCO₃, 11.1 glucose, and 2.16 CaCl₂, and 10 HEPES was initiated at a constant perfusion pressure of 60 mmHg. The buffer was maintained at 37°C and bubbled with 95% O₂ and 5% CO₂. Once cannulated, the hearts were allowed to acclimatize for 20 min before inducing a zero coronary flow global ischemia. After 30 min of ischemia, flow was restored and continued for 30 min. Coronary flow was recorded and coronary perfusate collected at baseline, immediately after reperfusion and following 10, 20, and 30 min of reperfusion (Fig. 1A). Nonischemic control hearts were continuously perfused for 60 min. Hearts treated with SP were given a 1-ml bolus of 1 mM SP 10 min before the initiation of ischemia as previously reported by others (21, 23).

**Rat LV tissue slice culture and hypoxia.** The LV tissue slice technique was used to examine the direct effects of SP on cell death independent of reperfusion. Also eliminated in this preparation is the possible influence of I/R-induced infiltration of neutrophils, which have been shown to be a source of oxidative stress in response to SP (13). As we have reported previously (12), rats were deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg) and the hearts removed, washed in cold sterile saline, and transferred to Joklik +10% FBS media. The LV plus septum were separated from the rest of the heart, filled with 2.5% agarose that was heated to 40°C to keep it liquefied, and inserted within a metal cylinder containing agarose. Once the agarose was solidified, a Bredel/Vitron Tissue Slicer was used to obtain 15 to 20 LV slices that were 250 to 300 μm in thickness.

The slices were incubated in calcium-free Joklik media at room temperature for 30 min and then transferred to fresh Joklik media containing 0.2 μM of CaCl₂ and incubated for 30 min in a cell culture incubator (37°C, 95% O₂ and 5% CO₂). Next, additional CaCl₂ was added to obtain a final concentration of 0.4 μM, and the slices were further incubated for 30 min. Depending on the experiment, the slices were divided randomly into groups with at least 3 slices per well as follows: 1) Normoxia (N); 2) Hypoxia (H); 3) Hypoxia treated with either 30 (H30), 100 (H100), or 300 (H300) nM SP (Tocris Bioscience, Minneapolis, MN); 4) 100 nM SP + NK-1R antagonist (10 μM L-732,138; Tocris Bioscience, Minneapolis, MN); or 5) 100 nM SP + AKT inhibitor (50 μM LY294002; Cell Signaling Technology, Danvers, MA). As stated above, 1 mM SP was administered as a 1-ml bolus to the isolated heart. In determining the SP concentration for the slices, we arbitrarily scaled this concentration down by the ratio of tissue weights of the isolated heart and the tissue slice weight per well or a factor of 10. Because this was an arbitrary reduction, three concentrations of 30, 100, and 300 nM were used. When the NK1 antagonist or the phosphoinositide 3-kinase AKT (p-AKT) inhibitor was used, the slices were incubated in Joklik media for 1 h instead of 30 min during the second addition of CaCl₂ since both require at least 60 min of incubation to establish effective inhibition. In all cases, SP was added to the Joklik media 10 min before transferring the slices to Waymouth media. SP was then added in an equivalent dose in the Waymouth media so as to maintain the same SP concentration before and during hypoxia.

Hypoxia was induced by incubating the LV slices in a deoxygenated, serum free Waymouth medium (1% O₂, 94% N₂ and 5% CO₂ at 37°C) for 45 min. Normoxic tissue slices were incubated in oxygenated Waymouth medium with serum (95% O₂, 5% CO₂ at 37°C) for 45 min. At completion of the experimental period, media from each well was collected, and the LV slices weighed. LV slices from each well were either snap frozen and stored at −80°C for subsequent biochemical analysis or fixed in 10% buffered formalin for 24 h at room temperature and then embedded in paraffin and sectioned to a 5 μm thickness for subsequent staining.

**Lactate dehydrogenase assay.** Lactate Dehydrogenase (LDH) is a cytosolic enzyme that is released from the cell as a result of sarcolemmal membrane rupture. To determine tissue injury, LDH was assayed in the coronary perfusate from the Langendorff experiments and in the media from the LV slice experiments by using a LDH cytotoxicity detection kit (Clontech, Mountain View, CA). For the LV tissue slice, the relative LDH activity in the culture media was normalized to the tissue slice weights.

**Western blots.** Total protein from LV tissue was extracted using T-PER buffer and a protease inhibitor cocktail (Thermo-Scientific, Rockford, IL). Protein samples were fractionated by SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies against AKT phosphorylated at Ser473 (1:2,000, in 5% BSA in TBS-T; Cell Signaling Technology, Danvers, MA) and GAPDH (1:1,000, in 5% nonfat milk in TBS-T; Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C, subsequently incubated with HRP-conjugated secondary antibodies (1:2,000 in 5% nonfat milk in TBS-T) for 1 h at room temperature and detected with an ECL Detection Kit (Thermo Scientific, Rockford, IL). The protein expression was quantified with ImageJ (National Institutes of Health) and adjusted to GAPDH.

**TdT-mediated dUTP-biotin nick end labeling.** Cell death was determined by TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay. LV tissue sections from each group were deparaffinized and rehydrated. TUNEL assay was performed using an In Situ Cell Death Detection Kit Roche, Mannheim, Germany) according to the manufacturer’s instructions. A negative control was obtained by incubating fixed and permeabilized tissue in Label solution only (i.e., without terminal transferase) instead of TUNEL reaction mixture, whereas a positive control was established by incubating fixed and permeabilized tissue with DNase I recombinant for 10 min at room temperature to induce DNA strand breaks, before the labeling procedure. In each section, all apoptotic cells were counted using a fluorescent microscope with the observer (S. Jubair) blinded as to the source of the section. Each section was randomly analyzed twice on separate days, and the results from the two readings averaged. The magnification was sufficiently high so as to distinguish and count only TUNEL positive stained cells (i.e., cardiomyocytes). We avoided counting TUNEL-positive endothelial cells in the epicardium, endocardium, and vessels. In addition, cell cutting damage artifacts to the TUNEL results were minimized as follows: the slices were first incubated in calcium-free Joklik media at room temperature for 30 min to allow for debris from cutting injured cells to wash out into the media before transferring the slices to fresh Joklik media; and the histological sections for TUNEL analysis were from the interior region of the slices after facing off 50 to 75 μm. The number of TUNEL-positive cells was divided by the section area (measured using a scanner GS-800) to obtain the number of apoptotic cells per square millimeter.

**Periodic acid-schiff staining.** Periodic acid acts upon the 1.2 glycol linkage of carbohydrates in tissue sections to produce aldehyde, which is highlighted using Schiff’s reagent. Accordingly, Periodic acid-Schiff (PAS) reagent can be used to detect tissue areas of abnormal sarcolemmal permeability to indicate early necrotic damage. PAS staining was performed using the PAS Staining System (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions. Briefly, tissue slides were deparaffinized, rehydrated, oxidized in 0.5% Periodic acid solution, and then immersed in Schiff reagent. Ten microscopic (200× magnification) fields per slide were randomly selected and photographed. The outline of each PAS positive area was traced and quantified using Image-Pro Plus 4.5 software; total PAS positive area was divided by the field area. Here, too, the reader (S. Jubair) was blinded as to the source of the tissue section being analyzed. The average percent PAS positive area of the 10 fields was obtained for each group.

**Identification of the NK-1R.** To determine whether cardiomyocytes possess the NK-1R, we isolated cardiomyocytes from adult mice as described previously (6, 14). Male C57BL/6 3-mo-old mice were sedated with Etomidate (10 mg/kg) and maintained with 3% inhaled isoflurane. Mice were then heparinized (5,000 units/kg) and the heart quickly removed and placed in ice-cold nominally Ca²⁺−free control solution (in mM) 133.5 NaCl, 4 KCl, 1.2
Fig. 1. A: ischemia-reperfusion (I/R) protocol schematic. B: coronary flow response to I/R in 3 groups of isolated rat hearts as follows: control; untreated I/R; and substance P (SP)-treated I/R. All values are means ± SD; n = 4 per group. *P < 0.05 vs. baseline (time = 0); **P < 0.05 vs. control at same time point; aP < 0.05 vs. SP I/R at same time point. C: percent change from baseline of lactate dehydrogenase activity (LDH) in the coronary perfusate during I/R in the 3 groups of isolated rat hearts. All values are means ± SE; n = 4. *P < 0.05 vs. control at same time point; **P < 0.05 vs. I/R at same time point.

MgSO₄, 10 HEPES, and 11 glucose. The aorta was cannulated and the heart mounted on a Langendorff apparatus and perfused at constant temperature 37°C and flow of 3 ml/min for 4 min with Ca²⁺-free control solution containing (in mM) 113 NaCl, 4.7 KCl, 0.6 KH₂PO₄, 0.6 Na₂HPO₄, 1.2 MgSO₄·7H₂O, 12 NaHCO₃, 10 HEPES, 30 taurine, 0.032 Phenol Red, 10 butanedione monoxime (BDM), and 5.5 glucose followed by an identical buffer containing additional enzymes [0.25 mg/ml Blendedzyme I (Roche), 0.14 mg/ml trypsin and 12.5 μM CaCl₂] for 8 min. The hearts were then removed, minced in digestion buffer, and pipetted until the tissues were completely dissociated. The cell suspension was rinsed in stop buffer (control buffer plus 5% of bovine serum albumin) to inactivate the enzymes. Once collected, the cells were lysed and combined with Laemmli buffer with β-mercaptoethanol (1:1); briefly sonicated (10 s) and boiled for 5 min before loading on a precast gel (Bio-Rad). Transfers were to a fluorochrome optimized PVDF membrane (Immobilon-FL, Millipore) since fluorescent Western blotting is more sensitive than chemiluminescence and we anticipated only low levels of the NK-1R receptor to be present on cardiomyocytes. Membranes were probed with an antibody to the NH₂ terminus of the NK-1R (1:500 dilution; Santa Cruz Biotechnology) and detected using a fluorochrome-conjugated secondary antibody and Odyssey imaging system (Li-Cor Biosciences).

Statistical analysis. Results are presented as means ± SE unless stated otherwise. For comparison between groups, paired data or one-way ANOVA was performed, followed by the Tukey post hoc test. Statistical significance was set at a P value <0.05.

RESULTS

Isolated heart I/R. In the isolated heart global I/R experiments, the ability of SP to induce coronary vasodilation and increase coronary flow during post-ischemia reperfusion was confirmed as shown in Fig. 1B. Upon reperfusion, coronary flow in the SP treated I/R (SP I/R) group was rapidly restored to control levels, whereas that in the untreated I/R group was significantly less than that in the control and SP I/R groups following 10, 20, and 30 min of reperfusion.

LDH is a cytosolic enzyme that is released from injured cells and used as a marker of cell death. The percent change from baseline LDH activity in the coronary efflux at the onset and subsequent 10-min intervals of reperfusion are presented in Fig. 1C. At the onset of reperfusion, LDH levels were found to be significantly increased in the I/R group by 240% above its baseline level, whereas in the SP I/R group the LDH level was increased by only 97% relative to its baseline value; the difference between these two groups at the onset of reperfusion was significant. During reperfusion, the percent change in LDH in the SP I/R group decreased to the same value as that in the control group while it progressively increased in the I/R group, indicating a continuing cell death.

LV slice experiments: effect of SP on cell death. To further study the effect of SP on cell death, we made use of the cultured LV tissue slice preparation. The LDH activity assay results normalized to the LDH activity value for the untreated hypoxia group are shown in Fig. 2. The normalized hypoxia group LDH activity value of 1.00 was greater compared with the H30 (0.73 ± 0.07; P = 0.056), H100 (0.69 ± 0.05; P <
0.05), and H300 (0.66 ± 0.10; P < 0.05) groups. LDH activity for the treated groups was not statistically different from that of the normoxic group (not shown in figure; 0.69 ± 0.05). These results further verify the ability of SP to attenuate hypoxia-induced cell death in the absence of reperfusion.

The TUNEL staining results for the five groups of LV slices are summarized in Fig. 3. As can be seen, incubation with increasing concentrations of SP resulted in a reduction in the number of TUNEL-positive cells (Fig. 3A). In Fig. 3B, the number of TUNEL-positive cells per unit area is expressed relative to that of normoxia group. There was a significant increase in apoptotic cells in the H group (1.7 ± 0.2) compared with the N and H300 (0.9 ± 0.1) groups. These results indicate the ability of SP to directly attenuate the number of hypoxia-induced apoptotic cells.

PAS staining was used to determine areas of increased sarcolemmal permeability, which reflects necrotic cell injury. In Fig. 3, C and D, the hypoxia group is seen to have a significant increase in PAS stained areas as compared with normoxia (8.1 ± 0.7% in H vs. 2.5 ± 0.2% in N), with the SP treated groups being significantly reduced compared with hypoxia (3.9 ± 0.3% in H100), and (4.6 ± 1.6% in H300). Accordingly, part of the cardioprotective properties of SP is its ability to attenuate necrotic cell injury induced by hypoxia.

**LV slice experiments: effect of SP on AKT activation.** When activated (phosphorylated), AKT promotes cell survival by its ability to inhibit cell death pathways. Incubation of hypoxic LV tissue slices with SP increased p-AKT in a concentration-dependent manner (Fig. 4, A and B). In Fig. 4B, p-AKT values are normalized to GAPDH and the fold changes relative to hypoxia are 1.35 ± 0.09 in H30; 1.90 ± 0.10 in H100; and 2.17 ± 0.47 in H300; the p-AKT values obtained for the SP 100 and SP 300 groups were significantly different from that in the untreated hypoxic group.

**LV slice experiments: role of AKT and NK-1R in SP-induced cell survival.** Due to the increased phosphorylation of AKT induced by SP, we performed the same experiments in the presence of an AKT inhibitor and a NK-1R antagonist. SP at a concentration of 100 nM was chosen based on the previous experiments. SP (H100) significantly reduced LDH levels in the media from hypoxic LV slices, with this effect prevented by AKT inhibition and NK-1R blockade independently (Fig. 5). Furthermore, SP (H100) also prevented increases in TUNEL-positive cells (Fig. 6, A and B) and PAS staining (Fig. 6, C and D), with these increases also being prevented by AKT inhibition and NK-1R blockade independently. Figure 7 indicates that both the AKT inhibitor and also the NK-1R antag...
 function to decrease, indicating that endogenous SP can provide a degree of protection from ischemic damage. Addition of exogenous SP to isolated hearts subsequently reduced LVEDP and improved contractile function, indicating that exogenous SP can provide additional protective effects over and above those provided by endogenous SP (23). This effect extends to the diabetic heart, which is known to have a deficit in SP, whereby the addition of exogenous SP reduced cell damage and improved cardiac function in an isolated heart model of postconditioning (19). Interestingly, although SP was reduced in the diabetic hearts, blockade of the NK-1R further increased cell damage and reduced LV function, indicating that the limited amount of endogenous SP remaining was still providing cardioprotection. These cardioprotective effects of SP were, at least in part, thought to be secondary to its potent coronary vasodilator actions, which could result in improved reperfusion and better regional distribution of myocardial flow (23, 27). Herein, we present data that SP also acts directly on myocardial cells to induce cell survival, independent of vasodilatory effects.

We initially performed I/R experiments using the isolated heart model of global I/R with a 30-min ischemia and 30-min reperfusion protocol in an attempt to make our findings relatable to the previous studies by Ustinova et al. (21) and Wang et al. (23), who used 20/30 min and 40/30 min I/R protocols, respectively. As with these previous studies we used SP at a concentration of $1 \times 10^{-6}$ M administered before ischemia. Using LDH in the coronary perfusate as a marker of cell damage, we noted that LDH levels were significantly decreased in the SP I/R group than in the untreated I/R group immediately upon reestablishment of coronary perfusion. Given that no flow was present during the ischemic period indicates that exogenous SP attenuated cell damage by its direct effects on myocardial cells rather than via vasodilatory actions. Interestingly, though, coronary flow was normalized by SP during the reperfusion period, confirming a previous report (23), and suggesting that the vasodilatory

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**DISCUSSION**

In the heart, SP is predominantly localized to nerves projecting to coronary arteries (3, 18, 24) and to a small population of coronary endothelial cells (16). Thus SP is ideally located to be rapidly released in response to changes in coronary pressure/flow such as occurs during an ischemic event. In fact, SP is released from the heart within 1 min of initiating ischemia (16) and continues to be produced at elevated levels even during a reperfusion period (23). Accumulating evidence from isolated heart experiments indicates that this endogenously released and also exogenously added SP is cardioprotective following I/R and during pre- and postconditioning interventions (19, 21, 23, 27). For example, Ustinova et al. depleted rat hearts of sensory nerve neuropeptides with capsaicin and then subjected those hearts to global ischemia (20 min) followed by 30 min of reperfusion. In comparison with noncapsaicin pretreated hearts, capsaicin-treated hearts had reduced recovery of heart rate, coronary flow, and LV developed pressure. Replacement of SP (1 nM to 1 µM) restored contractile function and coronary flow and the beneficial actions of SP could be prevented by NK-1R antagonism (21). Wang et al. reported in isolated heart experiments that blocking the NK-1R caused LV end diastolic pressure (LVEDP) to increase and contractile

**Fig. 4. A:** Western blot of activated AKT (Ser473 phospho-AKT) in hypoxic LV slices incubated with and without SP. The groups are as follows: H, H30, H100, and H300. B: group values were normalized to GAPDH and then expressed relative to the H group values. All values are means ± SE; n = 4. *P < 0.05 vs. H.

**Fig. 5.** LDH activity in the media of LV slices incubated before H with SP and AKT inhibitor or NK1 receptor antagonist (NK1 ANT). The media from hypoxic LV sections pretreated with 100 nM SP and AKT inhibitor or NK1R antagonist were not significantly different from the media of the hypoxia group. All values are means ± SE; n = 4. *P < 0.05 vs. H.
The effects of SP provided additional cell protection during the reperfusion period where LDH levels remained at control levels in the SP group in contrast with the untreated group where it continued to increase.

To further investigate these direct protective actions of SP and to avoid the influence of coronary flow on the cardiac tissue, we used a cultured LV tissue slice preparation (12), which has several advantages as follows: 1) myocardial cells retain their three-dimensional structural integrity, intercellular interactions, and extracellular attachments; 2) ability to perform multiple perturbations on tissue from an individual heart thereby minimizing biological variability; 3) relatively long-term effects of a perturbation can be investigated under highly controlled conditions; and 4) the myocardial response to perturbations is solely intrinsic in that it is void of factors, such as circulating inflammatory cells, cytokines, variations in the neuro-hormonal background, and variations in preload, afterload, and contractility. As in the isolated heart, exposure of the LV tissue slice to hypoxic conditions significantly increased LDH release into the media, which is indicative of cell damage. Addition of SP at the time of hypoxia significantly decreased the LDH levels in a dose-dependent manner. To confirm our LDH findings, we used PAS staining of LV tissue slice sections to delineate areas of early necrotic damage. The results clearly show that the PAS stained area in the hypoxia group was significantly larger than that in the normoxia group and the hypoxia group incubated with SP. Thus SP reduces cardiac necrotic cell damage during short-term hypoxia.

We also examined the effect of SP on apoptosis. TUNEL labeling showed a significant marked increase in apoptotic cell density in the hypoxia group than in the untreated normoxia and the hypoxia groups incubated with SP, clearly showing that SP attenuates hypoxia-induced apoptotic myocardial cell death. These results are consistent with previous reports of the anti-apoptotic effect of SP in hyperoxic-induced lung injury (7), retinal cells in diabetic rats (25), spinal cord injury (8),...
human tenocytes (2), bone marrow recovery after irradiation (1), intestinal tissue regeneration postirradiation injury (9), and sustained cardiac volume overload (15). Collectively, the LV tissue slice results demonstrate unambiguously that SP has a protective effect by acting directly on myocardial cells independent of its effect on coronary flow to increase cell survival following a hypoxic event.

A key molecule regulating cell survival is AKT. Phosphorylated AKT promotes cell survival and inhibits apoptosis by inactivating pro-apoptotic members of the Bcl-2 family, decreasing expression of caspases and increasing expression of anti-apoptotic Bcl-2 family members (17, 26). Because the media of normoxia group contained 10% FBS, which is known to induce AKT activation (20), we omitted the normoxia group from our statistical analysis and found p-AKT to be increased greater than twofold in the H300 group compared with the hypoxia without SP group. This clearly indicates that, with SP incubation, AKT was activated. We further confirmed the role of AKT in mediating the effects of SP via an AKT inhibitor (LY294002), which was able to prevent the beneficial actions of SP on cell damage, as determined by PAS staining and TUNEL labeling. Even though LY294002 has been shown not to be exclusively selective for the PI3Ks and could in fact act on other lipid kinases and additional unrelated proteins (5), it has been shown to act in vivo as a highly selective inhibitor of phosphatidylinositol 3 (PI3) kinase. Moreover, the concentration of 50 μM used herein has been shown to specifically abolish PI3 kinase activity ($IC_{50}$ = 0.43 μg/ml; 1.40 μM) and not inhibit other lipid and protein kinases such as PI4 kinase, PKC, MAP kinase, or c-Src (22). To investigate whether SP activation of AKT and subsequent cardioprotection occurs via the NK-1R, we used an NK-1R antagonist to treat LV slices incubated with SP. NK-1R blockade negated the ability of SP to phosphorylate AKT and to decrease the amount of ischemic damage, indicating the cardioprotective action of SP to be through the NK-1R. The results herein demonstrating that SP activates AKT via the NK-1R to enhance cardiac cell survival are schematically summarized in Fig. 9.

In a recent review by Dehlin and Levick (4), it was indicated that several groups of investigators were unable to identify...
NK-1R on adult cardiomyocytes using various indirect techniques, such as autoradiography, even though isolated neonatal rat cardiomyocytes have been shown to express mRNA for the NK-1R. Accordingly, this is the first study to demonstrate the existence of NK-1R on adult cardiomyocytes. This observation combined with the above results on apoptosis and necrosis indicate the ability of SP to directly provide cardiomyocyte protection against ischemic damage.

Our findings in myocardial cells corroborate the findings of Backman et al. (2) and Koon et al. (10) whereby the anti-apoptotic effect of SP was shown to be mediated by AKT in tenocytes and colonocytes, respectively. Accordingly, SP serves as a stimulus to protect cells against death and to promote survival. Nevertheless, it is important to note that there is one exception to the reports regarding the cardioprotective effects of SP. When the NK-1R was blocked in isolated hearts from magnesium-deficient rats, global ischemia-induced cardiac damage was reduced and cardiac performance was improved indicating that, in this model, SP promotes adverse effects (11). Whether this discrepancy was somehow related to the magnesium deficiency model remains to be determined.

In summary, the findings of this study demonstrate for the first time that SP exhibits cardioprotection independent of its effects on coronary flow. The percentage of apoptotic and necrotic cardiac cells were significantly decreased in hypoxic LV sections upon incubation with SP in a dose-dependent manner. This is also the first time that SP, acting via the NK-1R, has been shown to activate the AKT pathway in cardiac tissue and that the cardioprotective effect of SP is due to its ability to induce AKT activation. However, these cardioprotective effects of SP are based solely on experiments performed using ex vivo techniques, which lack the influx of inflammatory cells that occurs in vivo secondary to SP and other chemoattractant mediators. These inflammatory cells include the neutrophils that accumulate in the infarct zone during reperfusion. The fact that neutrophils have been shown to serve as a source of oxidative stress in response to SP (13), which in turn leads to enhanced development of cardiac lesions and contractile dysfunction, makes it unclear as to what the net in vivo effect of SP would be following ischemia reperfusion and whether SP administration has a long-term therapeutic potential. Nonetheless, the results herein indicate that there is a direct cardioprotective role of SP in the acute setting of cardiac ischemia.

REFERENCES


