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Grape Seed Proanthocyanidins Protect Cardiomyocytes From Ischemia and Reperfusion Injury Via Akt-NOS Signaling

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ABSTRACT

Ischemia/reperfusion (I/R) injury in cardiomyocytes is related to excess reactive oxygen species (ROS) generation and can be modulated by nitric oxide (NO). We have previously shown that grape seed proanthocyanidin extract (GSPE), a naturally occurring antioxidant, decreased ROS and may potentially stimulate NO production. In this study, we investigated whether GSPE administration at reperfusion was associated with cardioprotection and enhanced NO production in a cardiomyocyte I/R model. GSPE attenuated I/R-induced cell death [$18.0 \pm 1.8\%$ (GSPE, 50 μg/ml) vs. $42.3 \pm 3.0\%$ (I/R control), $P < 0.001$], restored contractility (6/6 vs. 0/6, respectively), and increased NO release. The NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 200 μM) significantly reduced GSPE-induced NO release and its associated cardioprotection [$32.7 \pm 2.7\%$ (GSPE + L-NAME) vs. $18.0 \pm 1.8\%$ (GSPE alone), $P < 0.01$]. To determine whether GSPE induced NO production was mediated by the Akt-eNOS pathway, we utilized the Akt inhibitor API-2. API-2 (10 μM) abrogated GSPE-induced protection [$44.3\% \pm 2.2\%$ (GSPE + API-2) vs. $27.0\% \pm 4.3\%$ (GSPE alone), $P < 0.01$], attenuated the enhanced phosphorylation of Akt at Ser473 in GSPE-treated cells and attenuated GSPE-induced NO increases. Simultaneously blocking NOS activation (L-NAME) and Akt (API-2) resulted in decreased NO levels similar to using each inhibitor independently. These data suggest that in the context of GSPE stimulation, Akt may help activate eNOS, leading to protective levels of NO. GSPE offers an alternative approach to therapeutic cardioprotection against I/R injury and may offer unique opportunities to improve cardiovascular health by enhancing NO production and increasing Akt-eNOS signaling. J. Cell. Biochem. 107: 697–705, 2009.

KEY WORDS: GRAPE SEED PROANTHOCYANIDIN EXTRACT; ISCHEMIA/REPERFUSION INJURY; CARDIOMYOCYTES; NITRIC OXIDE; Akt

The use of antioxidants to reduce oxidant injury during reperfusion is postulated to improve cardiac recovery from ischemia. Research in this field continues to explore various modalities and chemical entities that may attenuate oxidant activity at reperfusion and consequently reduce cardiac reperfusion injury [Ambrosio et al., 1995; Zweier and Talukder, 2006; Bolli, 2007]. Antioxidant compounds, which could be administered at reperfusion to mitigate tissue oxidant load, are now being considered as

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potential therapeutic interventions [Sato et al., 1999; Das and Maulik, 2006; Chang et al., 2007]. In that context, several herb-derived compounds that are extremely potent antioxidants [Rice-Evans, 2001] could be protective against acute oxidant stress. Grape seed proanthocyanidin extract (GSPE) is a mixture of several flavonoid constituents with potent antioxidant activity [Hung et al., 2000; Bagchi et al., 2003; Karthikeyan et al., 2007], and the consumption of grape skin- and grape seed-derived products has been linked to reduced incidence of cardiovascular disease [Das and Maulik, 2006].

In addition to the ability to scavenge reactive oxygen species (ROS) [Rice-Evans, 2004], GSPE has the ability to stimulate other cardioprotective pathways. Our previous work has shown that GSPE stimulates NO production in untreated cardiomyocytes in a dose-dependent manner. High-dose GSPE induced a high level of NO production that was associated with increased cardiomyocyte death while lower doses of GSPE were associated with a moderate increase in NO generation and did not induce cytotoxicity [Shao et al., 2006]. NO, which is a relatively stable free radical, acts as a signaling molecule in diverse physiological and pathological pathways. Recent studies indicate that augmenting NO bioavailability in acute oxidant stress models such as ischemia/reperfusion (I/R) is protective [Bolli, 2001; Schulz et al., 2004; Jones and Bolli, 2006]. Furthermore, the protective effect of polyphenolic antioxidants such as resveratrol in models of I/R has been attributed to increased NO production, as the nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) abrogates their protective effects [Bradamante et al., 2003; Thirunavukkarasu et al., 2007]. Thus, as an antioxidant that has the ability to stimulate NO release, GSPE may be a unique candidate for imparting protection against I/R injury.

NO release in numerous cellular models including cardiomyocytes has been intrinsically linked to regulation of NOS by the cell survival kinase Akt. Currently it is unknown if GSPE activates Akt, but several other antioxidants have been shown to confer improved cellular function via Akt activation [Toth et al., 2003; Goh et al., 2007]. In addition, the protective effect of resveratrol in a rat model of I/R has been shown, in part, to be due to activation of Akt [Goh et al., 2007]. It is therefore possible that GSPE induction of NO may occur via activation of the Akt-eNOS pathway [Hattori et al., 2002; Wallerath et al., 2002; Ha et al., 2008].

In the current study, we have investigated the effect of GSPE on both NO production and Akt activation in an acute oxidant stress model of ischemia and reperfusion. We hypothesized that acute GSPE treatment would confer protection from I/R injury to cardiomyocytes and that this protection would be due, at least in part, to the activation of Akt, leading to an increased production of NO during reperfusion.

MATERIALS AND METHODS

MATERIALS

Grape seed proanthocyanidin extract (GSPE), a water–alcohol extract from red grape seeds, was kindly provided by Prof. C.S. Yuan (Tang Center for Herbal Medicine Research, University of Chicago). The components of GSPE were analyzed using high liquid chromatography (HPLC) with gas chromatography (GC)-mass spectrometry (MS) by Adpen Laboratories, Inc. (Jacksonville, FL). The results of chemical analysis showed that GSPE is comprised of oligomeric proanthocyanidins (approximately 62.3%), monomeric flavanols (8.6% catechin, 8.7% epicatechin), and gallic acid (2.7%). API-2 (Akt/protein kinase B signaling inhibitor-2) was kindly provided by Prof. J.Q. Cheng (H. Lee Moffitt Cancer Center & research Institute, FL, USA). N_{ω}-nitro-L-arginine methyl ester (L-NAME) was purchased from Sigma (St. Louis, MO).

METHODS

Chick primary cardiomyocyte culture. Embryonic chick ventricular myocytes were isolated as previously described [Vanden Hoek et al., 1996]. Briefly, hearts of 10-day-old chick embryos were removed; the ventricles were minced and enzymatically digested with 0.025% trypsin (Invitrogen, Grand Island, NY) for 4–5 cycles with gentle agitation at 37°C. The resultant cell suspension was centrifuged, and the cell pellet was resuspended in media. Fibroblast contamination was reduced by pre-plating the cells for 45 min, and the purity of cardiomyocytes was confirmed as previously described [Vanden Hoek et al., 1996]. Cells (0.7×10^6) were plated onto glass coverslips (25 mm) and cultured in a humidified incubator for 3 days at which point the cells demonstrate synchronous contractions. Experiments were performed on day 4 or 5 using spontaneously contracting cells.

Simulated ischemia/reperfusion protocol. Synchronously contracting cells on glass coverslips were placed in a stainless steel flow-through chamber (1.2 ml volume, Penn Century Co., Philadelphia). The chamber was sealed with gaskets to minimize any O_2 exchange and mounted on an inverted microscope. The flow rate (0.25 ml/min), pH, temperature (37°C), and oxygen tension of the perfusate were controlled throughout the experiment. The standard perfusate for equilibration and reperfusion consisted of a buffered salt solution with a PO_2 of 149 torr, PCO_2 of 40 torr, pH of 7.4, 4.0 mM $[K^+]$, and 5.6 mM glucose. Simulated ischemic perfusate consisted of a buffered salt solution with 20 mM 2-deoxyglucose, 8.0 mM $[K^+]$, and no glucose. The ischemic solution was bubbled with 80% N_2 and 20% CO_2 to produce a $PO_2 < 3$ torr, PCO_2 of 144 torr, and a final pH of 6.8. We used an optical method of phosphorescence quenching (Oxyspot, Medical Systems, Inc., Greenvale, NY) to verify the conditions of ischemia [Vanden Hoek et al., 1997]. GSPE (50 μg/ml) was given throughout reperfusion in all applicable studies.

Video/fluorescent microscope. A Nikon TE 2000-U inverted phase/epifluorescent microscope was used for cell imaging. A charged-coupled device camera was used to monitor contractions and measure fluorescence over time in the same field of cells (approximately 70 μm × 90 μm). Fluorescent images were acquired from a cooled *Cool*-SNAP-ES camera (Photometrics, Tuscon, AZ) and changes in fluorescent intensity over time were quantified with MetaMorph® software (Molecular Devices Corp., Downington, PA) [Vanden Hoek et al., 1997].

Viability assay and contraction analysis. Cell viability was assessed by the fluorochrome propidium iodide (PI, 5 μM; Sigma) at

excitation 540 nm/emission 590 nm. PI is a fluorescent exclusion dye that binds to chromatin upon loss of cell membrane integrity. Cell death was quantified throughout the experiment in a select field of cells. At the end of each experiment, all cells in this field were permeabilized with digitonin (300 μM). Percentage cell death (PI uptake) was expressed as PI fluorescence relative to the maximal value seen after 1 h of digitonin exposure (100%). Cell contractions were assessed as previously reported [Vanden Hoek et al., 1996]. A return of contraction was indicated when contractions were seen throughout the field of cells.

Measurement of intracellular nitric oxide (NO) generation. Intracellular NO production was measured using the fluorescent dye 4, 5-diaminofluorescein diacetate (DAF-2 DA, 1 μM; EMD Biosciences, Inc., San Diego, CA). DAF-2 DA is a specific NO indicator that can penetrate rapidly into the cell, where it is hydrolyzed to DAF-2 by intracellular esterases. DAF-2 selectively traps NO, yielding fluorescent triazolofluorescein, which is measured at excitation 488 nm/emission 520 nm. Measurements were expressed as arbitrary units (a.u.) [Kojima et al., 1998].

Western blot analysis for Akt activation. Cardiomyocytes were subjected to experimental conditions and lysed at designated time points in cold lysis buffer (1% Triton-X100, 50 mM Tris pH 7.5, 40 mM β-glycerophosphate, 100 mM NaCl, 2 mM EDTA, 50 mM NaF, 200 μM Na_3VO_4, and 200 μM PMSF). Bradford assays (BioRad, Hercules, CA) were performed to determine protein concentration. Proteins (50 μg) were then separated on 10% SDS–PAGE gels (BioRad) and transferred to nitrocellulose (0.45 μm, Osmonics, Westborough, MA). Blots were blocked in 5% milk TBS-T for 1 h at room temperature followed by an overnight incubation at 4°C with primary antibody (Akt or phospho-Akt at Ser473; Cell Signaling) in 5% BSA TBS-T. Blots were then washed in several changes of TBS-T followed by 1 h incubation with the appropriate HRP-conjugated secondary antibody. Blots were washed as above and bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Densitometric analysis of Western blots was carried out using Quantity One Software (Bio-Rad, Richmond, CA), and results are presented as the ratio of phospho-Akt:total Akt.

DATA ANALYSIS

Each individual experiment on a field of ~500 cells represented an "n" of 1. All data are expressed as mean ± SEM. Statistical analysis was performed using commercially available statistic software (Sigma Stat 3.0). Data between groups recorded over an interval were compared using a two-way repeated measures ANOVA. When significant, Tukey's post-hoc test was performed for comparison between individual groups. A P-value <0.05 was considered statistically significant.

RESULTS

GSPE PROTECTS CARDIOMYOCYTES FROM ISCHEMIA/REPERFUSION (I/R) INJURY

To test whether GSPE protects cardiomyocytes against I/R-induced injury, cells were subjected to I/R and treated with GSPE during reperfusion. Cell death was assessed by propidium iodide (PI) uptake. We have previously reported that GSPE given at 50 μg/ml conferred optimal protection against oxidant-induced injury in cardiomyocytes [Shao et al., 2003], thus, this dose was used in the current study. As seen in Figure 1, GSPE (50 μg/ml) given at reperfusion (arrow) resulted in a significant decrease in cell death compared to I/R control [18.0 ± 1.8% vs. 42.3 ± 3.0% at 3 h reperfusion, respectively (n = 6/group, $P < 0.01$ to $P < 0.001$)]. Cell contractions also returned in GSPE-treated cells (6/6), but not in control I/R cells (0/6). These results indicate that GSPE treatment produced a profound and significant protection against I/R-induced cell death.

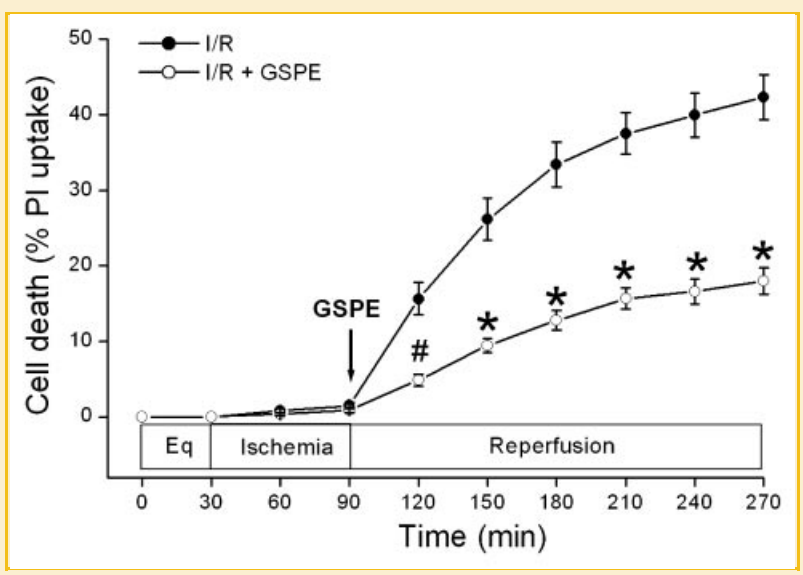

Fig. 1. Effect of GSPE on I/R-induced cell death. GSPE (50 μg/ml) given during reperfusion significantly attenuated cell death in cardiomyocytes. Data are expressed as means ± SEM, n = 6/group. $^{\#}P < 0.01$, $^{*}P < 0.001$ compared to I/R control cells.

THE ROLE OF NITRIC OXIDE (NO) IN GSPE-CONFERRED PROTECTION AGAINST I/R INJURY

NO has been postulated to play a protective role against cardiac I/R injury. As an NO indicator, DAF-2 DA has proven to be useful in detecting rapid NO generation in cardiomyocytes [Lebuffe et al., 2003]. We tested whether NO production, as measured by DAF-2 fluorescence, was increased with GSPE given during reperfusion and whether the increased NO release was associated with GSPE-conferred protection. Cells were loaded with DAF-2 DA (1 μM) and treated with GSPE (50 μg/ml). As seen in Figure 2A, GSPE-treated cells showed a marked increase in DAF-2 fluorescence during reperfusion as compared to I/R control cells [1,839 ± 49 a.u. vs. 810 ± 76 a.u. at 2 h reperfusion (n = 4/group, $P < 0.01$)]. To determine if the GSPE-induced increase in NO was generated through NOS, cardiomyocytes were pre-incubated with L-NAME (200 μM) for 2 h and continuously throughout the entire I/R protocol. This NOS inhibition partially abrogated the enhanced DAF-2 fluorescence seen in GSPE-treated cells (n = 4, $P < 0.05$), but had no effect on control I/R NO generation (Fig. 2A). Blocking NOS activation with L-NAME also at least partially abrogated the GSPE-mediated reduction in cell death [32.7% ± 2.7% GSPE + L-NAME vs. 18.0% ± 1.8% GSPE only at 3 h reperfusion (n = 6/group, $P < 0.01$); Fig. 2B], but did not significantly affect control I/R cell death [40.2% ± 4.5% L-NAME (n = 7) vs. 42.3% ± 2.9% I/R control at 3 h reperfusion (n = 4, NS; Fig. 2B]. These results suggest that the enhanced NO production at reperfusion is associated with GSPE-protection and may be mediated by enzymatic NOS.

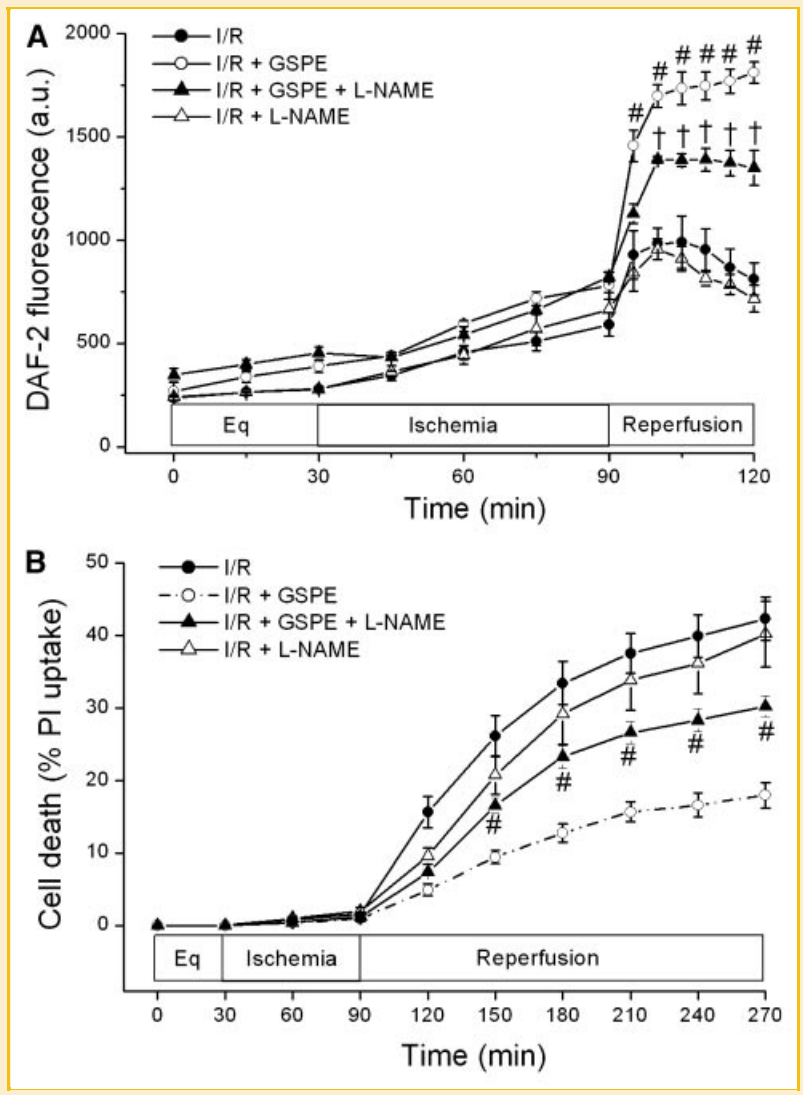

Fig. 2. Effect of NOS inhibition on GSPE-induced NO production and protection. Cells were pretreated with L-NAME (200 μM) for 2 h and also throughout the I/R protocol. GSPE (50 μg/ml) was administered only during reperfusion. A: Effect of NOS inhibition (L-NAME) on DAF-2 fluorescence in GSPE-treated cells subjected to I/R. DAF-2 fluorescence was significantly increased at the end of 30 min reperfusion in I/R control. GSPE (50 μg/ml) given at reperfusion further augmented the DAF-2 fluorescence, resulting in a twofold increase compared to I/R cells. Data are expressed as means ± SEM, n = 4/group; $^{\#}P < 0.01$. L-NAME (200 μM) concurrent with GSPE (50 μg/ml) treatment partially reversed the increased DAF-2 fluorescence seen in GSPE-treated cells (n = 4/group. $^{\dagger}P < 0.05$). B: Effect of L-NAME on the protective effect in GSPE-treated I/R cells. L-NAME partially abrogated the GSPE-induced cardioprotection. Data are expressed as means ± SEM, n = 6/group. $^{\#}P < 0.01$ versus GSPE-treated cells.

ROLE OF Akt IN GSPE-INDUCED NO PRODUCTION

Recent studies have shown that cardioprotection is associated with NO production following Akt-mediated eNOS activation in cardiomyocytes [Gao et al., 2002; Li et al., 2008]. Therefore, since we showed that NO generation is increased during GSPE-induced protection, we examined whether Akt was involved in the sustained reperfusion NO induced by GSPE. Cardiomyocytes were loaded with DAF-2 DA and treated with the Akt inhibitor API-2 (10 μM, 1 h pretreatment and throughout I/R). NO generation was measured in the presence and absence of GSPE (50 μg/ml, given at reperfusion). Figure 3A shows that blocking Akt activation partially abrogated the GSPE-induced increased in DAF-2 fluorescence [1,241 ± 60 a.u. GSPE + API-2 (n = 3) vs. 1,803 ± 97 a.u. GSPE alone (n = 3) at 2 h reperfusion, $P < 0.05$], implying that Akt plays a role in mediating GSPE-induced NO production in the context of I/R injury.

ROLE OF Akt IN GSPE-INDUCED CARDIOPROTECTION

Activation of Akt has been linked to protection against I/R injury in several cell types and animal models [Toth et al., 2003; Goh et al., 2007; Roviezzo et al., 2007]. To further examine the involvement of Akt in the protection conferred by GSPE, the effect of API-2 (10 μM) on cell death was examined. Figure 3B shows that inhibition of Akt abrogated GSPE-induced protection [44.3 ± 2.2% GSPE + API-2 (n = 4) vs. 28.0 ± 2.3% GSPE alone (n = 5) at 3 h reperfusion, $P < 0.01$]. However, Akt inhibition did not have a significant effect on control I/R cell death [48.3 ± 5.1% I/R + API-2 (n = 5) vs. 52.8 ± 5.8% I/R control at 3 h reperfusion (n = 5), NS].

Western blot analyses showed a drop in phospho-Akt during ischemia and I/R, but GSPE (50 μg/ml) given during reperfusion significantly increased phospho-Akt at Ser473 as compared to I/R control (n = 3, $P < 0.01$, Fig. 4). The Akt inhibitor API-2 blocked the

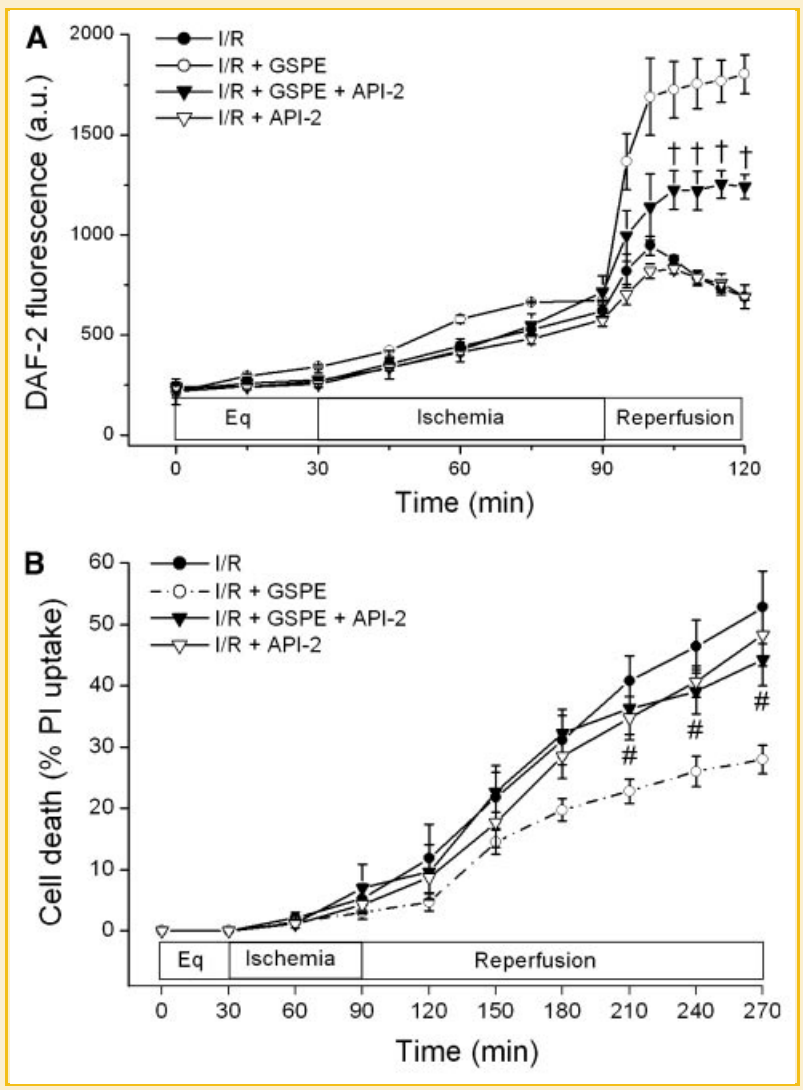

Fig. 3. Effect of Akt inhibition on GSPE-induced NO production and protection. Cells were pretreated with the Akt inhibitor API-2 (10 μM) for 1 h and also throughout the course of the experiment. GSPE (50 μg/ml) was given only during reperfusion. A: Effect of Akt inhibition (API-2) on DAF-2 fluorescence in GSPE-treated cells. Similar to Figure 2A, DAF-2 fluorescence was increased during reperfusion, and further augmented by addition of GSPE. API-2 partially reversed the GSPE-induced increase in DAF-2 fluorescence at reperfusion ($^{\dagger}P < 0.05$), while API-2 alone did not effect the DAF-2 fluorescence in the I/R control. B: Effect of Akt inhibition on GSPE-induced protection. API-2 partially abrogated the GSPE-induced protection. $^{\#}P < 0.01$ versus GSPE-treated cells.

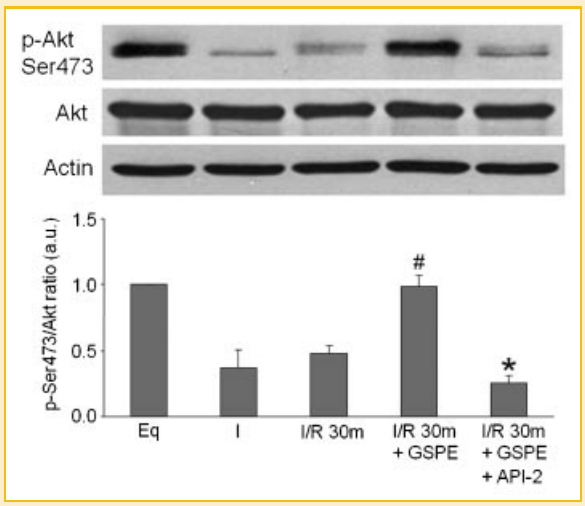

Fig. 4. Effect of I/R and GSPE treatment on Akt phosphorylation. A: Cardiomyocytes were subjected to experimental conditions, and cytosolic extracts were prepared from cells in the presence or absence of GSPE and API-2. GSPE (50 μg/ml) was given during reperfusion and API-2 (10 μM) was pretreated for 1 h and through I/R. The phosphorylation of Akt at Ser473 was analyzed by Western blot. Ischemia alone resulted in a decrease of phospho-Akt with a slight increase in phospho-Akt following 30 min reperfusion. GSPE caused a significant increase in phospho-Akt during reperfusion as compared to ischemia only or 30 min standard reperfusion. The GSPE-induced elevated phospho-Akt level was blocked by the addition of the API-2. Western blot shown is representative of three independent experiments. B: Densitometric analysis of Akt phosphorylation in GSPE-treated cells. Densitometric analysis of Western blots was carried out using Quantity One Software (Bio-Rad, Richmond, CA). Densitometric results are presented as the ratio of phospho-Akt/total Akt. Values are means ± SEM of three independent experiments. GSPE-induced a significant increase in phospho-Akt ($^{\#}P < 0.01$) during reperfusion, which was significantly attenuated by API-2 ($^{*}P < 0.001$).

increased phospho-Akt induced by GSPE ($P < 0.001$). These results support a role for phospho-Akt in GSPE-mediated cardioprotection in this model.

GSPE-INDUCED NO PRODUCTION VIA AN Akt–eNOS PATHWAY

Because inhibition of either Akt or NOS resulted in only a partial blockade of protective NO generation, we examined whether the activation of Akt and eNOS could be acting in the same or separate pathways. For these studies, cells were treated concurrently with L-NAME (200 μM) and API-2 (10 μM) following the above protocols. As shown in Figure 5, as with either inhibitor alone, a partial inhibition of GSPE-induced NO generation was seen [1,404 ± 96 a.u. GSPE + API-2 + L-NAME (n = 5) vs. 1,812 ± 51 a.u. GSPE alone (n = 5) at 2 h reperfusion, $P < 0.05$]. These results suggest that Akt may be important in the activation of eNOS-mediated NO production, which leads to GSPE-induced protection. Overall, these results indicate that GSPE may induce NO through a mechanism involving the activation of an Akt–eNOS pathway.

DISCUSSION

In the current study, we demonstrate that acute treatment with grape seed proanthocyanidin extract (GSPE) significantly reduces cardiomyocyte death and leads to the return of spontaneous contractions in our model of ischemia and reperfusion (I/R) injury. The protective effect of GSPE was associated with an enhanced, sustained NO generation at reperfusion that was abrogated by NOS inhibition. GSPE given during reperfusion also increased phosphorylation of the survival kinase Akt at Ser473 following I/R while inhibition of Akt by API-2 decreased phospho-Akt levels and the GSPE-induced protection. These results suggest that GSPE protects cardiomyocytes from I/R injury, at least in part, by increasing Akt

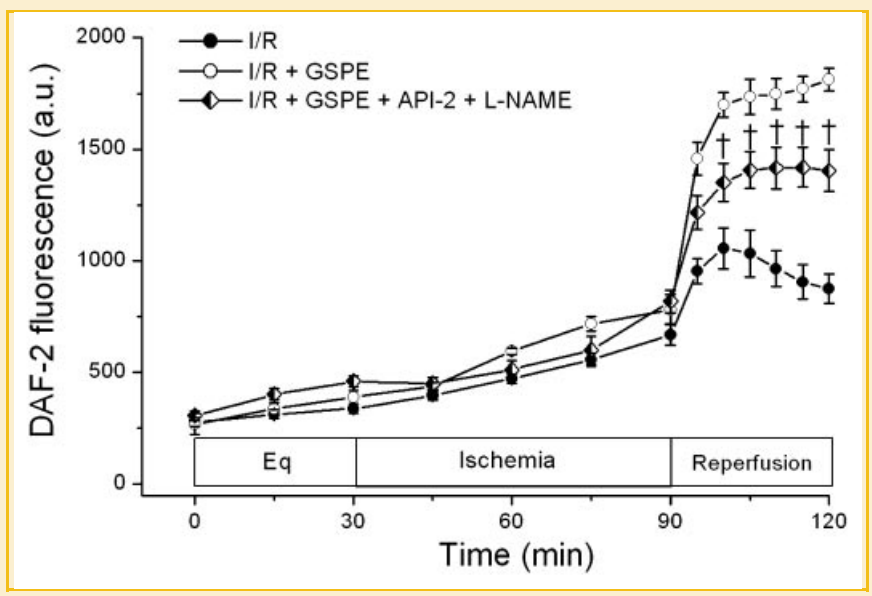

Fig. 5. Effect of NOS and Akt inhibition on NO production. Cells were pretreated with both API-2 (10 μM) and L-NAME (200 μM) as previously described; GSPE was given at reperfusion only. Concurrent treatment with both inhibitors partially inhibited the augmented DAF-2 fluorescence induced by GSPE. Data are expressed as means ± SEM. $^{\dagger}P < 0.05$ versus GSPE-treated cells.

phosphorylation, which leads to the maintenance of an elevated level of NO.

NO has been implicated as a cardioprotective agent during I/R injury in various cell types and models [Gao et al., 2002; Brunner et al., 2003; Li et al., 2008] and its release in reperfused tissues has been attributed to the activation of NOS, especially eNOS [Bolli, 2001; Schulz et al., 2004]. In one study, primary cardiomyocytes and murine embryonic fibroblasts were protected against I/R induced cell death when physiological levels of NO (1% in N_2) were given exogenously during ischemia [Iwase et al., 2007]. Furthermore, a NO donor given throughout I/R has also been shown to be protective in a mouse heart Langendorff model [Bell et al., 2003]. In an in vivo rat model, a statin given just prior to reperfusion exerted an acute protective effect against myocardial reperfusion injury by increasing the phosphorylation of eNOS via the PI3-K/Akt pathway [Wolfrum et al., 2004]. In the current study, we extend our prior work that showed GSPE stimulated a concentration-dependent NO production in untreated cardiomyocytes [Shao et al., 2006] by investigating whether GSPE-induced NO generation at reperfusion was protective in our cardiomyocyte model and examined the potential mechanism behind this protection.

Our studies show that NO increases in non-GSPE treated cardiomyocytes at reperfusion, corroborating the results reported in other studies [Bolli, 2001; Schulz et al., 2004]. We also find that GSPE given during reperfusion results in an enhanced, sustained NO level that is significantly higher than that of the control I/R group (Fig. 2A). These results suggest that GSPE stimulates an augmented NO response and that this response is generated through a NOS isoform (i.e., an enzyme-dependent mechanism) since L-NAME inhibits it. Furthermore, we find that the protective effect of GSPE during I/R is dependent on this increased NO release as NOS inhibition with L-NAME eliminates the protective effect of GSPE. Taken together, these results suggest that GSPE induces an enzyme-dependent NO production in reperfused cardiomyocytes.

The exact mechanism by which NO exerts its protective effects in the context of I/R has not been completely elucidated. NO may act by reducing intracellular calcium concentration and thereby reducing cell death [Mery et al., 1993] or it may act by reducing myocardial contractility and myocardial oxygen consumption that results in attenuated mitochondrial ROS formation [Xie et al., 1998; Scott et al., 2001]. NO may also mediate mitochondrial K_{ATP} channel opening, which has been associated with reduced reperfusion injury [Sasaki et al., 2000]. NO also augments antioxidant protection by forming intracellular antioxidants (nitrosothiols and glutathione) [Ronson et al., 1999] and by reducing ROS release through inhibition of NADPH oxidase activity [Fujii et al., 1997]. In addition, eNOS over-expression induced in endothelial tissue improved reperfusion injury in a transgenic mouse model [Jones et al., 2004], underlining the fact that enzymatic NO attenuates reperfusion injury. Further studies elucidating how GSPE induced NO regulates I/R injury are ongoing.

The mechanism by which GSPE induces NO production in cardiomyocytes has not been elucidated in previous studies. However, GSPE has been shown to stimulate NO formation in various cell types such as endothelial and glial cells [Roychowdhury et al., 2001; Berti et al., 2003; Vitseva et al., 2005], and it had been postulated that GSPE may induce NO formation by the redox-sensitive activation of Src kinase with the subsequent PI3-kinase/Akt-dependent phosphorylation of eNOS in the endothelium [Anselm et al., 2007]. Additional studies have shown that inhibiting Akt blocks GSPE-induced activation of eNOS in human umbilical vascular endothelial cells [Edirisinghe et al., 2008] and this mechanism was also shown to exist in adult rat cardiomyocytes [Xu et al., 2005]. Furthermore, polyphenolic compounds found in red wines have also been shown to induce protection via increased NO release, which is due to a PI3-K-dependent activation of Akt [Ndiaye et al., 2005]. PI3-K is a redox-sensitive kinase; thus, it may be activated through changes in intracellular ROS levels, leading to eNOS activation and increased NO release [Ndiaye et al., 2005]. In this study, we showed that GSPE's cardioprotective effects are associated with increased phospho-Akt signaling at Ser473 within 30 min of administration and that these effects are blocked with the Akt inhibitor API-2 (Fig. 4). API-2 also blocked GSPE increases in NO (Fig. 3A). These results suggest that the cardioprotective effect of GSPE may lie with its ability to induce NO production via Akt signaling. Thus, our results show that the increase in, or maintenance of, phospho-Akt is critical to GSPE-induced cardioprotection and that phospho-Akt is, in part, responsible for the activation of eNOS. In contrast to our work, a recent study by Engelbrecht et al. demonstrated that GSPE attenuated PI3 K/Akt phosphorylation in a colon cancer cell line (CaCo2) and enhanced apoptosis. However, this study did not show the effects of GSPE on Akt in normal terminally differentiated cells. Thus, GSPE may have divergent effects on Akt signaling depending on cell phenotype, but the mechanisms for this remain unknown [Engelbrecht et al., 2007].

In conclusion, GSPE conferred protection in our cardiomyocyte model of I/R which was associated with increases in NO and phospho-Akt. GSPE induced cardioprotection was abrogated in the presence of the NO inhibitor L-NAME and the Akt inhibitor API-2 suggesting that the Akt-NOS pathway is important for GSPE induced cardioprotection. We believe the increased NO concentration produced a cytoprotective environment, resulting in reduced cell death and restoration of contractile function following reperfusion. Thus, the protection conferred by GSPE against reperfusion injury appears to be mediated by increased NO production during reperfusion via the activation of Akt and an enzymatic isoform of NOS.

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