Niacin-bound chromium enhances myocardial protection from ischemia-reperfusion injury

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A significant number of in vitro, animal and human clinical investigations have demonstrated the biological and pharmacological safety of niacin-bound chromium, which improved cardiovascular functions, promoted lean body mass, and prevented diabetes in experimental animals and humans by promoting the action of insulin and modulating protein, fat, and carbohydrate metabolism (26). Withania somnifera (ashwagandha, also known as Indian ginseng) has been extensively used as a valuable drug in Ayurveda, the traditional medicine of India. Evidence has also shown that Withania somnifera can modulate the lipid peroxidative end products, such as malondialdehyde, the endogenous antioxidants, such as glutathione, and the antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (32). The pentose sugar ribose has been shown to significantly increase the rate of de novo adenine nucleotide synthesis by bypassing the rate-limiting step in the oxidative pentose phosphate pathway and thus elevating the phosphoribosyl pyrophosphate levels, leading to the synthesis of ATP (39). Replenishing the tricarboxylic acid cycle components via amino acids rather than by fatty acids would increase ATP production, with positive effects on cellular metabolism. Moreover, oral supplementation with mixed essential amino acids protects the heart against ischemia-reperfusion injury, reducing both the extent of the infarct size and the magnitude of apoptotic cell death (34).

EF consisting of niacin-bound chromium, d-ribose, ashwagandha, and selected amino acids has been indicated for energy production, fat metabolism, the induction of nitric oxide, and several other clinically important stress-related proteins. AMP kinase (AMPK) is a serine/threonine protein kinase that has been described as a sensor for the energy state in various cell types. The rise in the level of AMP, coupled with the decrease in ATP, activates phosphorylated AMPK (p-AMPK), to maintain ATP level. Thus AMPK is known as the “guardian of energy status” (11). Over a decade ago, it was shown that whole body heat shock activates a powerful endogenous protective mechanism that significantly improved myocardial salvage after prolonged ischemia and reperfusion injury to the heart (23). A characteristic feature of this heat shock response is the expression of a family of proteins known as heat shock proteins (HSPs) (23). Evidence has shown a direct correlation with EF in both male and female rats. Aortic flow, maximum first cycle components via amino acids rather than by fatty acids would increase ATP production, with positive effects on cellular metabolism. Moreover, oral supplementation with mixed essential amino acids protects the heart against ischemia-reperfusion injury, reducing both the extent of the infarct size and the magnitude of apoptotic cell death (34).

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between HSP production and the degree of myocardial protection (4).

To address whether this hypothesized cardioprotective action will be achieved by supplementation of EF, we orally administered EF to male and female Sprague-Dawley rats for 30, 60, and 90 days, followed by measurements of ATP, creatine phosphate (CP), p-AMPK, and HSP levels after ischemia-reperfusion. To correlate biochemical and functional changes in the myocardium, heart rate, coronary and aortic flow, maximum first derivative of developed pressure (dP/dt max), and left ventricular developed pressure were also determined during myocardial ischemia-reperfusion injury. In the present study, the protective effects of EF on the heart after ischemia-reperfusion injury have been confirmed both biochemically and functionally.

MATERIALS AND METHODS

Animal maintenance and treatment. The animal protocol used in this study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Connecticut Medical Health Center. All animals received humane care in compliance with the principles of the laboratory animal care formulated by the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985). Sprague-Dawley male and female rats weighing 175–199 g (6–7 wk old) were used for the study and were divided into four groups: 1) control; 2) EF (40 mg/kg in 0.5 ml of water as suspension/day) treatment for 30 days; 3) EF (40 mg/kg in 0.5 ml of water as suspension/day) treatment for 60 days; and 4) EF (40 mg/kg in 0.5 ml of water as suspension/day) treatment for 90 days. EF, a combination of niacin-bound chromium (0.45%), D-ribose (10.71%), caffeine (22.76%), ashwagandha extract (10.71%), and selected amino acids, including phenylalanine, taurine, and glutamine (55.37%), was obtained from InterHealth Nutraceuticals. Age-matched controls were used for 30, 60, and 90 days, respectively. EF was orally administered to rats for 90 consecutive days. All hearts were then subjected to 30-min ischemia followed by 2-h reperfusion.

Isolated working heart preparation. Rats were anesthetized with pentobarbital sodium (80 mg/kg ip; Abbott, Baxter Health Care, Deer Field, IL), and heparinized (500 IU/kg iv) (Elkins-Sinn, Cherry Hill, NJ). After a sufficient depth of anesthesia was ensured, a thoractomy was performed and hearts were perfused in the retrograde Langendorff mode at 37°C at a constant perfusion pressure of 100 cmH2O (10 kPa) for a 5-min washout period (18). The perfusion buffer used in this study consisted of a modified Krebs-Henseleit bicarbonate buffer (KHB) (in mM: 118 NaCl, 4.7 KCl, 1.7 CaCl2, 25 NaHCO3, 1.2 KH2PO4, 1.2 MgSO4, and 10 glucose). The Langendorff preparation was switched to the working mode after the washout period, as previously described (8). The working mode was introduced by switching the flow to the left atrium from the aortic root with a constant preload of 17 cmH2O and an afterload of 100 cmH2O.

At the end of 10 min, after the attainment of steady-state cardiac function, baseline functional parameters were recorded. The circuit
was then switched back to the retrograde mode, and hearts were perfused for 15 min with KHB buffer. The hearts were then subjected to global ischemia for 30 min and then 2 hrs of reperfusion. The first 10 min of reperfusion was in the retrograde mode to allow for postischemic stabilization and thereafter in the antegrade working mode to allow for assessment of functional parameters, which were recorded at 30-, 60-, 90- and 120-min reperfusion.

Cardiac function. Aortic pressure was measured by using a pressure transducer (Micro-Med) connected to a sidearm of the aortic cannula, and the signal was amplified by using a Heart Performance Analyzer Model 400 (Micro-Med) (30). Heart rate (HR), left ventricular developed pressure (LVDP), and dP/dtmax were all derived or calculated from the continuously obtained pressure signal (35). Aortic flow was measured by using a calibrated flowmeter (Gilmore Instrument, Barrington, IL), and coronary flow was measured by timed collection of the coronary effluent dripping from the heart.

Infarct size estimation. At the end of reperfusion, a 1% (wt/vol) solution of triphenyl tetrazolium chloride in phosphate buffer was infused into the aortic cannula for 1 min at 37°C. The hearts were excised and stored at −70°C. Sections of frozen heart were fixed in 10% formalin, placed between two coverslips, and digitally imaged with the use of a Microtek Scan Maker 600Z. To quantitate the areas of interest in pixels, NIH Image version 5.1 (a public domain software package) was used. The infarct size was quantified and expressed in pixels.

Western blot analysis. To quantify the abundance of the HSPs such as HSP-25, -70, -32, and p-AMPK, we performed Western blot analysis using various specific primary antibodies. Heart tissues from each treatment group were homogenized and suspended (50 mg/ml) in sample buffer (10 mM HEPES (pH 7.3), 11.5% sucrose, 1 mM EDTA, 1 mM EGTA, diisopropylfluorophosphate (DFP), 0.7 mg/ml pepstatin A, 10 mg/ml leupeptin, and 2 mg/ml aprotinin). The homogenates were centrifuged at 3,500 rpm, and the cytosolic fractions were used for protein analysis. The total protein concentration was determined by using a bicinchoninic acid protein assay kit (Pierce, Rockville, IL). The cytosolic proteins were run on polyacrylamide electrophoretic gels (SDS-PAGE) typically using 12% (acrylamide-
Effects of EF on myocardial function. There was no significant difference in heart functions (LVDP, in mmHg), heart rate (in beats/min), dP/dt max (in mmHg/s), coronary flow (in ml/min), and aortic flow (in ml/min) before the induction of ischemia in the EF-treated hearts in comparison with untreated control hearts. In general, there was no significant difference between EF and controls in heart rate and coronary flow (Fig. 1). After ischemia, on reperfusion, the values of all the functional parameters were decreased in all four groups. A significant increase in LVDP after 120 min of reperfusion was found in both male and female rats after 30 days of treatment (Fig. 1A), but at 60 (Fig. 1B) and 90 (Fig. 1C) days, a significant difference in LVDP was found in females but not in males compared with control animals. Significant increases in dP/dt max and aortic flow were observed during reperfusion in EF-treated rats. After 120 min of reperfusion, postischemic values of dP/dt max were significantly increased from the ischemic-reperfused control values in both male and female rats for...
30 days [2,067 (SD 210) and 1,578 (SD 71) vs. 1,603 (SD 264) and 1,237 mmHg/s (SD 50)], 60 days [1,752 (SD 75) and 1,647 (SD 44) vs. 1,499 (SD 172) and 1,231 mmHg/s (SD 52)], and 90 days [1,780 (SD 180) and 1,669 (SD 119) vs. 1,482 (SD 217) and 1,261 mmHg/s (SD 56)], respectively (Fig. 1, A–C).

Similarly, aortic flow significantly increased in both male and female rats for 30 days [11.25 (SD 2.82) and 1.5 (SD 0.54) vs. 1.92 (SD 1.38) and 0.23 ml/min (SD 0.41)], 60 days [8.42 (SD 1.02) and 6.85 (SD 1.01) vs. 0.92 (SD 0.34) and 0.5 ml/min (SD 0.55)], and 90 days [8.4 (SD 0.58) and 6.67 (SD 0.82) vs. 0.65 (SD 0.40) and 0.5 ml/min (SD 0.55)], respectively (Fig. 1, A–C). The cardioprotective effects of EF-treated animals were demonstrated by a significant recovery of postischemic myocardial function.

**Effects of EF on myocardial infarct size.** Infarct size (percentage of infarct vs. total area at risk) was significantly higher in the control hearts subjected to 30-min ischemia followed by 2-h reperfusion compared with the hearts that were not subjected to ischemia-reperfusion protocol (almost at the baseline level; data not shown). The values were significantly reduced after EF treatment at 30 days [47.9 (SD 4.7) vs. 30.6% (SD 2.3) in male rats; 33.9 (SD 2.1) vs. 20.9% (SD 1.8) in female rats; Fig. 2A], 60 days [48.1 (SD 6.9) vs. 26.5% (SD 4.4) in male rats; 32.9 (SD 2.1) vs. 20.5% (SD 3.1) in female rats; Fig. 2B], and 90 days [44 (SD 1) vs. 22% (SD 2) in male rats; 32 (SD 1) vs. 15.6% (SD 2.7) in female rats; Fig. 2C] compared with the ischemia-reperfusion control (Fig. 2). Again, a significant difference in the infarct size was found in control male rats when compared with female control rats at 30 days [47.9 (SD 4.7) vs. 33.9% (SD 2.1)], 60 days [48.1 (SD 6.9) vs. 32.9% (SD 2.1)], and 90 days [43.9 (SD 1.1) vs. 32.2% (SD 1.2)] after 30 min of ischemia and 2 h of reperfusion, respectively.

**Effects of EF on HSP.** EF treatment for 30 days in both male and female rats significantly enhanced the expression of HSP-25 (Fig. 3), -32 (Fig. 4), and -70 (Fig. 5), the significance was sustained for the 60- and 90-day treatment, respectively, when compared with control animals.

**Effects of EF on phosphorylation for AMPK.** EF also significantly enhanced the phosphorylation of AMPK in all three groups compared with the control group. As shown in Fig. 6, A and B, phosphorylation of AMPK was increased in both male and female EF-treated rats at 30, 60, or 90 days.

**Effects of EF on ATP and CP levels.** Myocardial ATP levels increased by 7, 58, and 59% (Fig. 7A), and CP levels increased by 17, 31, and 59% (Fig. 8A) after 30, 60, or 90 days of EF supplementation, respectively, in male rats compared with control animals. Similarly, the ATP levels increased by 12, 57, and 80% (Fig. 7B), and CP levels increased by 16, 61, and 63% after 30, 60, or 90 days (Fig. 8B) of EF treatment in female rats.

**DISCUSSION**

This study shows that oral supplementation of EF in both male and female rats provided cardioprotection as evidenced by super-
D-ribose has been known to rebuild downregulated targets, and thus helps in maintaining the ATP/AMPK, leading to the phosphorylation of a large number of proteins. A decrease in the AMP-to-ATP ratio triggers the activation of AMPK, resulting in increased ATP levels. Reports show that once activated, AMPK increases energy supply by triggering the ATP-generating pathways and decreasing the energy demand by reducing the ATP-consuming process, which regulate the cellular energy status, resulting in cardioprotective effects (11). AMPK is highly expressed and activated in cellular responses that increase the AMP-to-ATP ratio (10). In our present study, the increase in the myocardial ATP and CP levels in both male and female rats supplemented with EF might be due to activation of one or more of three regulatory mechanisms, including increased p-AMPK levels, ribose supplementation, or amino acid supplementation, which acts directly/indirectly as a source of energy that regulate ATP production and utilization (16).

HSPs or stress proteins perform a range of functions, including cytoprotection and the intracellular assembly, folding, and translocation of oligomeric proteins (29). EF supplementation increases the expression of HSPs like HSP-70, -32 (HO-1), and -25. Donnelly et al. (6) observed a reduction in infarct size by the induction of HSPs when rat hearts are exposed to 35 min of left coronary artery occlusion in the intact animal model. Nonetheless, the identification of compounds able to induce the HSPs without inducing a full stress response is always a viable therapy. In our present study, EF supplementation improved functional recovery and decreased infarct size in male and female rats compared to nontreated animals. These observed effects may be due to increased expression of HSPs. Reports have demonstrated protective effects of HSPs in preventing heart damage after ischemia-reperfusion injury (37). Moreover, ribose supplementation has been shown to result in enhanced recovery of ATP after myocardial ischemia and to improve diastolic functional parameters (31). Ashwagandha has been found to preserve left ventricular function after ischemia-reperfusion injury (15). These protective effects may be attributable to the induction of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) and HSPs in the heart. Evidence shows that hearts isolated from transgenic mice that have been engineered to express human HSPs in the myocardium have shown greatly improved functional recovery, with decreased infarct size after the experimental induction of ischemia and reperfusion (14, 27). HO-1 is known to cleave heme to yield carbon monoxide and the protective antioxidant biliverdin (7). Studies by Liesy and Tomar (17) demonstrated a direct role of bilirubin resulting from the heme oxygenase activation as a physiological protector against oxidative injury (17). In addition, the induction of heme oxygenase is coupled to the synthesis of ferritin (20), which is known to be a cytoprotective antioxidant (1). HO-1 knockout mice showed enhanced infarct formation after exposure to hypoxia, indicating an important protective role for this HSP (38). HSP-70 is found to protect cardiac cells against simulated ischemia or thermal stress in vitro (2) as well as in a model of ischemia-reperfusion injury via the suppression of inflammatory cytokines (9). Sammut et al. (28) have also shown that HSP-70 upregulation may protect the mitochondrial energy metabolism in the injured heart by repairing the ion channels under stress conditions. HSP-25 (also called as...
HSP-27 has been shown to occur relatively higher in heart tissue (12), is known to help in restoring the redox balance (5), and plays a regulatory role in muscle contraction (13). Moreover, the functional recovery of EF-treated animals after ischemia might be due to its amino acid content. Pasini et al. (22) have shown that long-term treatment with amino acids reduces the increase of diastolic pressure during ischemia and improves the recovery of developed pressure in reperfusion without influencing the preischemic hemodynamics of isolated hearts. In addition, amino acids are precursors of important molecules like glutathione, which potentially may be responsible for ischemic myocardial protection (22). Several studies have also reported that L-arginine acts as precursor of nitric oxide, which plays an important role in ischemia-reperfusion injury by increasing postischemic blood flow (33).

In conclusion, this formulation (EF) demonstrated long-term safety as well as exhibiting significant cardioprotective ability by increased energy production, improved cardiac function, and reduced infarct size, which proves its ability for the treatment of heart disease in humans.

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REFERENCES