Cardioprotective mechanisms of *Prunus cerasus* (sour cherry) seed extract against ischemia-reperfusion-induced damage in isolated rat hearts

Istvan Bak, Istvan Lekli, Bela Juhasz, Norbert Nagy, Edit Varga, Judit Varadi, Rudolf Gesztelyi, Gergo Szabo, Levente Szendrei, Ildiko Bacskay, Miklos Vecserynes, Miklos Antal, Laszlo Fesus, Francois Boucher, Joel de Leiris, and Arpad Tosaki

Departments of 1Pharmacology, 2Pharmaceutical Technology, 3Anatomy, and 4Biochemistry, Health Science Center, University of Debrecen, Debrecen, Hungary; and 5University of Joseph Fourier, Grenoble, France

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The onset of severe ischemia in the myocardium sets into motion a series of pathological events that continue until the tissues die. These changes begin seconds after ischemia and occur because the supply of oxygen is insufficient to support oxidative phosphorylation in the cardiac tissue. Although reperfusion is an absolute criteria for the survival of ischemic tissues, the notion has developed that it is not without hazard, and reperfusion-induced pathological changes may occur and further aggravate the previously ischemia-induced damage, thus indicating that ischemia- and reperfusion-induced injury may not be separately treated by various pharmacological interventions. In the search of the mechanisms of ischemia-reperfusion-induced pathways that may be amenable to manipulation, a number of potential candidates have been identified and have been the subject of many investigations. It is highly probable that a number of interaction mechanisms combine to determine the damage caused by ischemia-reperfusion in the myocardium, and a variety of such triggers have been postulated, including ionic disturbances and ion channels (30, 69), fatty acid metabolism (35), α- and β-adrenergic receptors (67), various gene expression (53, 60, 64), platelet-activating factor (6), endothelin (50), nitric oxide (24), heme oxygenase-1 and carbon monoxide (5, 61), and free radicals (15, 30). It has been also shown that ischemia and reperfusion of the myocardium result in an activation of various pathways including caspase cascade, and it is hypothesized that a degree of caspase inhibition could be related to the recovery of postischemic cardiac function (4, 31, 57, 62, 66).

It is generally suggested that diet has a major role in the development chronic diseases, such as coronary heart disease, cancer, obesity, Type 2 diabetes, hypertension, and cataracts (17, 33, 42a, 71). Bioactive compounds are extranutritional constituents that are typically naturally occurring in small quantities in plant products (16). Most notably, fruits, vegetables, legumes, and seeds are high in fiber, relatively high in flavonoids, anthocyanidins, polyphenols, free unsaturated fatty acids, and low in saturated fat, transfat, and dietary cholesterol (33, 71). There is epidemiologic evidence demonstrating a protective role in diets high in fruits and their seeds, vegetables, legumes, and fish oil on various cancers and cardiovascular diseases. Thus it is generally accepted that recommendations of fruits, natural extracts from plants, vegetables, and less processed staple foods provide substantial protection against the development of various diseases (33). Thus it is reasonable to assume that sour cherry (*Prunus cerasus*) seed extract containing various bioactive components could play an important role in cardiac protection, and, as a consequence, the ischemia-reperfusion-induced damage in cardiac function, the incidence of reperfusion-induced arrhythmias, apoptotic cell death, and infarct size could be reduced. Postischemic cardiac function, infarct size, and the incidence of arrhythmias were compared between the sour cherry seed extract-fed and the untreated control groups. Caspase-3 activity was reduced in ischemic-reperfused hearts obtained from rats pretreated with sour cherry seed extract, indicating that a reduction in caspase-3 activity could be related to the recovery, at least in part, of postischemic cardiac function. The number of apop-
totic cells was also reduced. Thus the use of sour cherry seed kernel extract may be a new therapeutic tool for the treatment of ischemic heart diseases.

MATERIALS AND METHODS

Preparation of isolated heart. Male Sprague-Dawley rats (280–360 g body wt) were used for all studies. Animals were anesthetized with pentobarbital sodium (60 mg/kg ip), and heparin (500 IU/kg) was injected intravenously. After 20 sec of heparin injection, hearts were excised and placed in ice-cold perfusion buffer. The isolated working heart model was used, and this has been described in detail previously (55). The aorta was cannulated, and Langendorff perfusion (100 cm of water, 10 kPa) was initiated. During the Langendorff perfusion, the pulmonary vein was cannulated for conversion of the perfusate was ejected spontaneously at a rate of 45–65 ml/min (measured by a calibrated flow meter) through the aortic cannula against a hydrostatic pressure of 100 cm of the perfusion buffer (10.0 kPa). The perfusion buffer consisted of a modified Krebs-Henseleit bicarbonate buffer containing (in mM) 118 NaCl, 5.8 KCl, 1.8 CaCl₂, 25 NaHCO₃, 0.36 KH₂PO₄, 1.2 MgSO₄, and 5.0 glucose. Global ischemia was imposed by clamping the atrial and aortic canulas. All animals received humane care in compliance with the “Principles of Laboratory Animal Care,” formulated by the National Society for Medical Research, and the “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. 86-23, Revised 1996). The use of animals in our experiments was approved by the Institutional Animal Care and Use Committee (IACUC).

Induction of ischemia and reperfusion. After aerobic perfusion of the heart, both the aortic outflow and pulmonary inflow lines were clamped at a point close to the origin of the aortic and pulmonary canulas, thus the global ischemia could then be maintained for any desired period by clamping the inflow line. Reperfusion could be initiated by unclamping and removing the occluders. An epicardial ECG was recorded by a computer acquisition system (ADInstruments, PowerLab, Castle Hill, Australia) throughout the experimental period with the use of two silver electrodes attached directly to the heart. ECGs were also recorded for the incidence of ventricular fibrillation (VF) and ventricular tachycardia (VT) during the first 2 min of “nonworking” Langendorff reperfusion. The heart was considered to be in VF if an irregular undulating baseline was present on the ECG. VT was defined as five or more consecutive premature ventricular complexes, and this classification included repetitive monomorphic VT, which is difficult to dissociate from rapid VT. The heart was considered to be in sinus rhythm if normal sinus complexes in a regular rhythm were present on the ECG. The first 10 min of 2-h reperfusion period was initiated by “nonworking” Langendorff mode. If VT and VF developed and the sinus rhythm did not spontaneously return within the first 2 min of “nonworking” Langendorff reperfusion, hearts were electrically defibrillated by a defibrillator using two silver electrodes and 15-V square-wave pulse of 1-ms duration and reperfused. Then, after the first 10 min of Langendorff reperfusion, hearts were further reperfused by switching to “working” mode for an additional 110 min. Heart rate (HR), coronary flow (CF), aortic flow (AF), and left ventricular developed pressure (LVDP) were recorded and measured after 60 min and 120 min of reperfusion.

 Determination of infarct size. Hearts for infarct size measurement were perfused at the end of each experiment, with 25 ml of 1% triphenyltetrazolium chloride solution in phosphate buffer (88 mM Na₂HPO₄ and 1.8 mM NaH₂PO₄) via the side arm of the aortic cannula and then stored at −70°C for later analysis. Frozen hearts were sliced transversely (59) in a plane perpendicular to the apical-basal axis into 2- to 3-mm-thick sections, weighted, blotted dry, placed in between microscope slides, and scanned on a Hewlett-Packard Scanjet 5p single-pass flatbed scanner (Hewlett-Packard, Palo Alto, CA). With the use of the NIH Image 1.61 image processing software, each digitalized image was subjected to equivalent degrees of background subtraction and brightness/contrast enhancement for improved clarity. Infarct zones of each slice were traced, and the respective areas were calculated in terms of pixels (20). The areas were measured by computerized planimetry software, and these areas were multiplied by the weight of each slice. The results were then summed up to obtain the weight of the risk zone (total weight of left ventricle, in mg) and the infarct zone (in mg). Infarct size was expressed as the ratio (in %) of the infarct zone to the risk zone.

 Measurement of caspase-3 activity by immunohistochemistry. The free-floating sections of the heart were first incubated with biotinylated goat anti-caspase-3 antibody (diluted 1:1,000, Sigma, St. Louis, MO) for 2 days at 4°C. The immunological and immunocytochemical characteristics of antibody have been published earlier (36). Sections were then transferred into a solution of biotinylated rabbit antibody (diluted 1:200, Vector, Burlingame, CA) for 1 h at room temperature, then into avidin-biotinylated-peroxidase complex (diluted 1:100, Vector) for 4 h at room temperature, and was completed with a diaminobenzidine chromogen reaction (34). Before, sections were kept in 10% normal goat serum (Vector, Burlingame, CA) for 50 min. All incubations were performed under continuous gentle agitation, and all of antibodies were diluted in 10 mM PBS (pH 7.4) to which 0.1% Triton X-100 and 1% normal rabbit serum (Vector) were added. Sections were mounted on gelatin-coated slides and covered with Permount neutral medium (Fluka, Buchs, Switzerland).

 Determination of cardiomyocyte cell apoptosis. The formaldehyde-fixed left ventricle was embedded in paraffin, cut into transverse sections (4 μm thick), and deparaffinized with a graded series of xylene and ethanol solutions. Immunohistochemical detection of apoptotic cells was carried out using transferase-mediated dUTP nick-end labeling (TUNEL) in which residues of digoxigenin-labeled dUTP are catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase, an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA (41). The incorporated nucleotide was incubated with a sheep polyclonal anti-digoxigenin antibody followed by a FITC-conjugated rabbit anti-sheep IgG as a secondary antibody, as described by the manufacturer’s instructions (Roche, Branchburg, NJ). The sections (n = 5) were washed in PBS three times, blocked with normal rabbit serum, and incubated with mouse monoclonal antibody recognizing-α-sarcomeric actin (Sigma), followed by staining with tetramethyl rhodamine isocyanate-conjugated rabbit anti-mouse IgG (200:1 dilution, Sigma). The fluorescence staining was viewed with confocal laser microscopy (Fluoview, Olympus, Tokyo, Japan). For the quantitative purpose, the number of TUNEL-positive cardiomyocytes was counted on 100 high-power fields (magnification, ×600) from the endocardium through the epicardium of the midportion of the left ventricular free wall in five sections from each heart (28). Representative confocal images show α-sarcomeric actin-positive endothelial cells (red staining in their cytosol), which are negative for TUNEL staining (absence of green staining in the nucleus) as well as those positive for TUNEL staining (magnification, ×600).

 Experimental time course. We performed these studies to assess the therapeutic value of sour cherry seed extract to improve the recovery of myocardial function in ischemic-reperfused hearts. Sour cherry seeds were dried, and the wall was removed. The kernel was then ground and extracted with n-hexane by Soxhlet extractor. The solvent was evaporated under vacuum, resulting in the oil fraction (fraction 1) of the kernel (32–36%). The remaining (64–68%) solid fraction (fraction 2) was dried, and the oil-free kernel extract was used for the analyses of its cardiovascular effects. UV, infrared, and gas chromatography/mass spectrometry analyses in composition with HPLC showed that sour cherry seed kernel extract contains 32–36% of...
vegetable oils, including triglycerides, oleic acids, α-tocopherol, tocopherols, and tocopheryl-like components. The components of 64–68% of the solid fraction of sour cherry seed kernel extract are various bioactive structures, including 2–4% of cyanides, 1–3% of polyphenols, 1–4% of flavonoids, 1–3% of vegetable acids, 1–2% of pro- and anthocyanidines, 1% of trans-resveratrol, 1% of stilbenes, and 1% of catechins (63). Before the isolation of hearts, rats were treated orally with 1, 5, 10, and 30 mg·kg⁻¹·day⁻¹ of sour cherry seed extract for 14 days. Age-matched control rats received a daily dose of saline solution (0.9% of NaCl) for 14 days. Hearts were excised and perfused with a drug-free buffer according to the Langendorff method for a 5-min washout period. During the washout period, the pulmonary vein was cannulated as described earlier. The studies had two single objectives. The first was to ascertain whether sour cherry seed kernel extract can reduce the incidence of reperfusion-induced arrhythmias and improve myocardial function in ischemic-reperfused hearts. To achieve this, we used five groups of hearts, respectively: hearts from group 1 were perfused aerobically and subjected to 30 min of ischemia, followed by 120 min of reperfusion, and rats from groups 2–5 were treated with various doses of sour cherry seed kernel extract (1, 5, 10, or 30 mg·kg⁻¹·day⁻¹) for 14 days, and the hearts were then isolated and subjected to 30 min of ischemia, followed by 120 min of reperfusion. Preischemic values for heart function were registered before the induction of ischemia, and postischemic cardiac function was measured during reperfusion.

The second objective was to study whether the extract can attenuate the infarct size, caspase activity related to apoptotic cell death. The samples for infarct size and caspase activity measurements were taken after 30 min of ischemia followed by 120 min of reperfusion.

Statistics. HR, CF, AF, LVDP, infarct size, and apoptotic cells were expressed as means ± SE. A two-way analysis of variance was first carried out to test for any differences in mean values between groups. If differences were established, the values of the drug-treated groups were compared with those of the drug-free group by Dunnett’s test. A different procedure, because of the nonparametric distribution, was used for the distribution of discrete variables, such as the incidence of VF and VT. An overall χ-square test was used to compare individual groups.

RESULTS

Figure 1 shows the incidence of reperfusion-induced VF (Fig. 1A) in isolated hearts obtained from rats treated with 0, 1, 5, 10, and 30 mg/kg of sour cherry seed extract for 14 days, respectively, and subjected to 30 min of ischemia, followed by 120 min reperfusion. Thus, with the doses of 1, 5, 10, and 30 mg/kg of sour cherry seed kernel extract, a significant dose-dependent reduction in the incidence of reperfusion-induced VF was detected from its control value of 92% to 92% [not significant (NS)], 75% (NS), 50% (NS), and 17% (P < 0.05), respectively. In the reduction of the incidence of reperfusion-induced VT (Fig. 1B), the same protection was observed.

The protection against the development of reperfusion-induced VF and VT in rats treated with various doses of sour cherry seed kernel extract reflected in the improvement in the recovery of postischemic cardiac function. Thus, in hearts obtained from rats treated with 10 and 30 mg/kg of sour cherry seed kernel extract, a significant recovery in CF, AF, and LVDP was observed compared with that of drug-free control values (Table 1). For instance, after 30 min of ischemia followed by 60 min of reperfusion (Table 1), AF was significantly increased from its drug-free ischemic-reperfused control value of 9.5 ± 0.7 to 22.0 ± 1.5 (P < 0.05) and 27.2 ± 2.1 ml/min (P < 0.05) with the concentrations of 10 and 30 mg/kg of extract, respectively. The same extent of postischemic recovery was observed in CF and LVDP. Interestingly, HR was not substantially and significantly changed using various doses of the extract. The lower doses of sour cherry seed kernel extract (1 and 5 mg/kg) failed to significantly improve the postischemic recovery in HR, CF, AF, and LVDP (Table 1).

Figure 2 shows the results and representative pictures of infarct size above each bar in untreated and drug treated ischemic-reperfused rat hearts. The infarct size was significantly reduced from its drug-free control value of 38.3 ± 1.3 to 26.5 ± 2% (P < 0.05) and 21.8 ± 1.8% (*P < 0.05), in hearts obtained from rats treated with 10 and 30 mg/kg of sour cherry seed kernel extract for 14 days, respectively, and subjected to 30 min of ischemia followed by 120 min of reperfusion. Lower concentrations of the extract (1 and 5 mg/kg) failed to significantly reduce infarct size (data not shown).

Figure 3, A–D, shows caspase-3 activities in hearts subjected to ischemia-reperfusion from rats treated with sour cherry seed kernel extract for 14 days. Caspase activity, using immunohistochemistry, was reduced in treated subjects, indicating by a reduction in brown staining intensity (Fig. 3, A–D) in the...
myocardium. Figure 3, E–H, also shows the results of ischemia-reperfusion-induced apoptosis. It is clearly shown that numbers of apoptotic cells, indicated by green staining, were significantly reduced in hearts treated with the kernel extract of sour cherry seed (Fig. 3, G and H) compared with the extract-free ischemic-reperfused control group (Fig. 3F). The numerical values of apoptotic cells are shown (Fig. 3I).

DISCUSSION

In the present study, the kernel extract of sour cherry seed was applied before the induction of ischemia, thereby slowing the rate of development of ischemic injury so that, at the time of reperfusion, the myocardium in the treated group is less severely injured. Because the extent of damages, including postischemic cardiac function, is proportional to the severity of the antecedent ischemic period, it is impossible to ascertain whether the observed protection by sour cherry seed extract is a direct consequence of a reduction of reperfusion-induced damage or is secondary to some of its antiischemic effect. There are some salient features of our study. First, the kernel extract of sour cherry seed, as a natural product originated from Prunus cerasus, was found to provide cardiac protection against ischemia-reperfusion-induced damages as evidenced by the reduction of the incidence of ventricular arrhythmias and infarct size. Second, the extract increased the recovery of postischemic cardiac function, including CF, AF, and LVDP. Third, sour cherry seed kernel extract reduced caspase-3 activities. Finally, the extract significantly reduced apoptotic cell death by preventing the development of ischemia-reperfusion-induced functional damages. It is well known that reperfusion of the ischemic myocardium is associated with apoptotic cell death in concern with DNA fragmentation. It is also well known that a large portion of cell loss during myocardial ischemia and reperfusion occurs through necrosis (56), and there is currently increasing interest in the possibility that cardiac cell death could also occur through apoptosis via various signal transduction mechanisms (27, 40, 43, 49, 54, 58). Many apoptotic mechanisms have been proposed for the development of apoptosis, but TNF-α (43), protein kinase C (65), p53 (29), p38 mitogen-activated protein kinase (46 – 47), and caspases (19) have been suggested and frequently cited as important signaling mechanisms. For the first time, the results of our study thus showed that beneficial effects of the kernel extract obtained from sour cherry seed are due to its ability to reduce caspase-3 activation, which is linked, at least in part, with the reduction of apoptotic cell death. Our results show that reperfusion could induce apoptosis, including caspase activation, and it is possible that ischemia initiated the signal for apoptosis that took a couple of hours to develop. It was not the aim of the present investigation to ascertain to what extent that apoptosis and necrosis (infarcted area) individually contribute to the development of myocardial infarction (and probably, both of them contribute to the development), and a so-called “necro-apoptotic” mechanism contributes to the development of ischemia-reperfusion-induced injury (42). However, we demonstrate that under our experimental circumstances, the extract significantly reduces the extent of myocardial infarction and improves postischemic cardiac function, providing evidence that one of the key signaling pathways controlling apoptosis could mediate, at least in part, ischemia-reperfusion-induced injury. Furthermore, the results of our study suggest that, although proapoptotic signaling plays an important role in the development of reperfusion-induced damage, caspase inhibition by itself may not afford alone a complete protection against postischemic damage in our model. There are currently abundant data to indicate that different signal mechanisms contribute to apoptosis leading to postischemic cardiac failure, but it is reasonable to believe that different and multiple mechanisms, rather than a single factor, could significantly contribute to the development of cardiac cell death (8–9, 22, 44, 48). This is supported and well explained by the finding of

Table 1. Cardiac function in untreated and sour cherry seed extract-treated hearts

<table>
<thead>
<tr>
<th>Extract</th>
<th>Before Ischemia</th>
<th>After 60 min of Reperfusion</th>
<th>After 120 min of Reperfusion</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HR CF AF LVDP</td>
<td>HR CF AF LVDP</td>
<td>HR CF AF LVDP</td>
</tr>
<tr>
<td>Extract</td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>306±8</td>
<td>26.5±1.1</td>
<td>17.7±0.3</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>308±6</td>
<td>27.5±0.9</td>
<td>17.5±0.3</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>321±8</td>
<td>26.8±1.2</td>
<td>17.3±0.2</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>313±5</td>
<td>27.8±1.1</td>
<td>17.2±0.2</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>303±7</td>
<td>28.3±1.1</td>
<td>17.5±0.3</td>
</tr>
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</table>

Values are means ± SE; n = 6 hearts/group. Comparisons were made to the time-matched extract-free control group. HR, heart rate (in beats/min); CF, coronary flow (in ml/min); AF, aortic flow (in ml/min); LVDP, left ventricular developed pressure (in kPa). *P < 0.05.

Fig. 2. Effects of different doses of sour cherry seed extract on infarct size in isolated rat hearts subjected to 30 min of ischemia, followed by 120 min of reperfusion. Isolated hearts were obtained from rats treated orally with 10 and 30 mg/kg of sour cherry seed extract, respectively, for 14 days. Values are means ± SE; n = 6 hearts/group. *P < 0.05 compared with untreated age-matched, drug-free control value. Representative slice of infarct size is shown above each bar. White areas indicate infarcted tissues using triphenyl-tetrazolium chloride staining.
Ma et al. (46) showing that the administration of a p38 MAPK inhibitor completely blocked p38 MAPK activation, but this concentration failed to completely prevent the development of apoptosis-induced cell death in the myocardium. Of course, other apoptotic signal mechanisms not specifically discussed in the present study, e.g., TNF-α (10, 43, 49), p53 (29), Akt (18), glucose, and cellular ATP contents, also may play an important role in the development of apoptosis-induced cardiac damages (40, 58).

It has been well known for many decades that VF is responsible for sudden cardiac death, and a large portion of cell loss during ischemia and reperfusion occurs through necrosis.
Some of the current possibilities over the ability of the extract to prevent ischemia-related damage. The results obtained with the application of sour cherry seed kernel extract in isolated hearts do not prove that the presence of bioactive natural molecular structures that originated from the extract are present in the circulation and tissues of intact animals at biologically active concentrations after oral administration. To clarify the above assumption, additional pharmacokinetic studies are needed.

GRANTS

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CARDIAC PROTECTION WITH SOUR CHERRY SEED EXTRACT


