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Cardioprotection with the Parrodiene 2,4,6-Octatrienal and Its Potassium Salt through Activation of the Akt-Bcl-2 Survival Pathway

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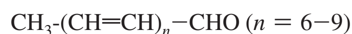
A study was undertaken to determine the cardioprotective effects of parrodienes prepared from the feather pigments of parrots (*Ara macao*). Adult male Sprague–Dawley rats were divided into three experimental groups and perfused with KHB buffer with or without treatment of 2,4,6-octatrienal (**1**) (50 mM) or its potassium salt (**2**) (50 mM). All hearts were then subjected to 30 min ischemia followed by a 2 h reperfusion. Ischemia/reperfusion resulted in a significant amount of tissue injury, cardiomyocyte apoptosis, and depression in hemodynamic functions. Parrodiene treatment prevented the development of myocardial injury after ischemia/reperfusion. Western blot analysis indicated that **1** and **2** reduced the oxidative stress, induced the expression of Bcl-2, and caused increased phosphorylation of Akt. These agents also reduced myocardial ischemic reperfusion injury presumably by reducing oxidative stress and activating the survival signal through the Akt-Bcl-2 pathway.

Parrodienes[1] are a family of natural and synthetic polyunsaturated linear aldehydes and derivatives with 2–9 conjugated double bonds. While aldehydes with 6–9 double bonds were extracted from parrot plumage and tissues, compounds with 2–6 double bonds have been synthesized following literature methods[2] by condensation of acetaldehyde with crotonaldehyde or by self-condensation of crotonaldehyde. Natural parrodienes are formed from carotenoids when parrots consume the latter in their diet. These carotenoids are chemically polyunsaturated aldehydes and are metabolized to different compounds with different colors that are responsible for the feather color of parrots.

In recent years, the general structure of the parrodienes has been demonstrated by spectroscopic analysis[3,4] and shown to bear the following structural formula that is similar to carotenoids:



$$CH_3\text{-}(CH{=}CH)_n{-}CHO \; (n = 6{-}9)$$

Structural similarities between parrodienes and carotenoids lead to some common biological properties such as antioxidant and anti-inflammatory activities.[1,5] However recent studies have shown that parrodienes also possess properties that differ from those of carotenoids.[5] Parrodienes exhibit an antioxidant property by inhibiting the formation of reactive oxygen species and by blocking the formation of hydroxyl radicals, which are the most reactive products among the reactive oxygen species that produce the greatest damage to cells and tissues.[5]

It is known that ischemia/reperfusion-induced injury causes an imbalance in oxygen supply, resulting in oxidative stress in the myocardium. Reports are available in the literature to show that carotenoids have a cardioprotective effect against ischemia reperfusion injury.[6] As parrodienes possess antioxidant and other activities and also possess structural similarities to carotenoids, it seems likely that parrodienes might also demonstrate cardioprotective properties.

In this study, we have evaluated the pharmacological properties of two synthetic parrodienes, 2,4,6-octatrienal (**1**) and 2,4,6-octatrienoate K^+ salt (**2**) (Figure 1). The results of the present investigation have revealed significant cardioprotection with these parrodienes, as evidenced by improvement in cardiac function and reduction in infarct size. Biochemical analysis has also revealed the generation of the survival signal against ischemia reperfusion injury by inducing the activation of Akt and Bcl-2 after pretreating the heart with synthetic **1** and **2**.

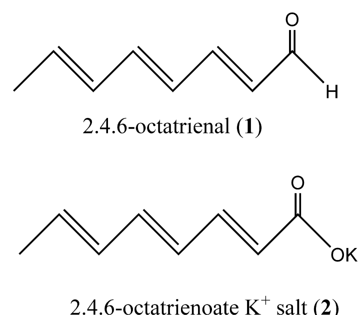


Figure 1. Structures of 2,4,6-octatrienal (**1**) and 2,4,6-octatrienoate K^+ salt (**2**).

Results and Discussion

Effects of 1 and 2 on Left Ventricular Function. The hearts from three groups of rats were isolated and perfused in the working mode. The treated hearts were subjected to 15 min of preperfusion with **1** or **2** followed by 30 min of global ischemia and 120 min of reperfusion. On the other hand, the control group of hearts was subjected to 30 min of ischemia followed by 120 min of reperfusion. The nonischemic control groups of hearts were perfused with KHB buffer for the same time period without subjecting them to the ischemia reperfusion protocol. The left ventricular functional parameters such as the left ventricular developed pressure (LVDP), the first derivative of maximum left ventricular developed pressure (LVDP max d*p*/d*t*), the aortic flow (AF), the coronary flow (CF), and the heart rate during the experimental procedure were all recorded. As the treatments were performed after recording the baseline data, no changes were found in baseline functional parameters between the groups. As was expected during postischemic reperfusion, the values of all functional parameters were decreased in all the groups compared to their respective baseline value. Rats from **1**- or **2**-treated groups showed significant recovery

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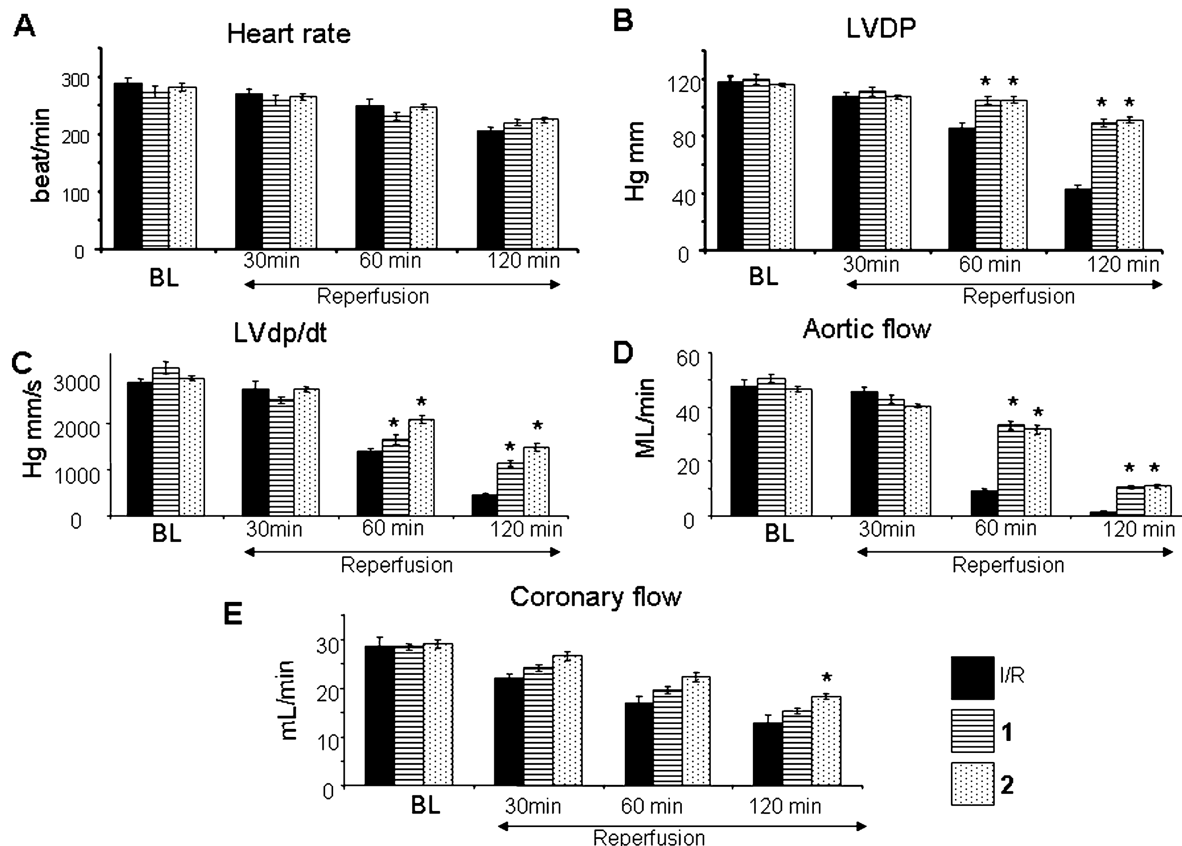

Figure 2. Effect of parrodienes on the postischemic ventricular performance. Isolated hearts were perfused with KHB only or KHB + 50 mM parrodienes [octatrienal (**1**)/octatrienal K^+ salt (**2**)] for 15 min followed by 30 min of global ischemia and 120 min of reperfusion in working mode. Heart rate (A), left ventricular developed pressure (B), first derivative of left ventricular developed pressure (C), aortic flow (D), and coronary flow (E) were monitored. The results shown are the mean ± SEM of $n = 6$ in each group. Comparisons were made to the values of test compound-free control group. $*p < 0.05$.

of postischemic function. Figure 2 shows that the LVDP was significantly higher in the case of **1**- or **2**-treated rats. Similarly, there were significant improvements of LVDP max d*p*/d*t* and AF. Any significant changes were not shown in the case of heart rate and coronary flow between the two groups.

Effects 1 or 2 on Myocardial Infarct Size. After 30 min of ischemia followed by 120 min of reperfusion, myocardial infarct size was determined by the TTC (triphenyl tetrazolium chloride) method. As shown in Figure 3, 30 min of ischemia followed by 120 min of reperfusion produced 34.2 ± 1.9% infarct in the case of the control group, but perfusion with the parrodienes significantly reduced the infarct size to 16 ± 2.6% (**1**) and 15 ± 3.5% (**2**).

Effects of 1 or 2 on Cardiomyocyte Apoptosis. As shown in Figure 4, cardiomyocyte apoptosis determined by the TUNEL method was about 32 ± 2.3% at the end of reperfusion in the case of the ischemic control group. There were very few apoptotic cells (4%) in the hearts perfused with the KHB buffer without being subjected to ischemia and reperfusion (nonischemic control), and in the case of **1** and **2** treatment significantly reduced the number of apoptotic cells to 15 ± 1.4% and 13.6 ± 1.3%, respectively.

Effects of 1 or 2 on the Induction of Cytochrome *c* and Caspase 3. In order to confirm the prevention of cardiomyocyte apoptosis by **1** and **2**, the activities of cytochrome *c* and caspase 3 in the cytosolic fraction were determined. Figure 5 shows that the cytochrome *c* level was significantly increased in the cytosolic fraction after ischemia/reperfusion in the case of the ischemic control group, but perfusion with **1** and **2** prevented the release of cytochrome *c* in the cytosolic fraction. Activity of pro-caspase 3 was significantly reduced in the ischemic reperfused myocardium, but for the **1**- and **2**-treated groups the amount of pro-caspase 3 was higher. Similarly, the activity of cleaved caspase 3 was

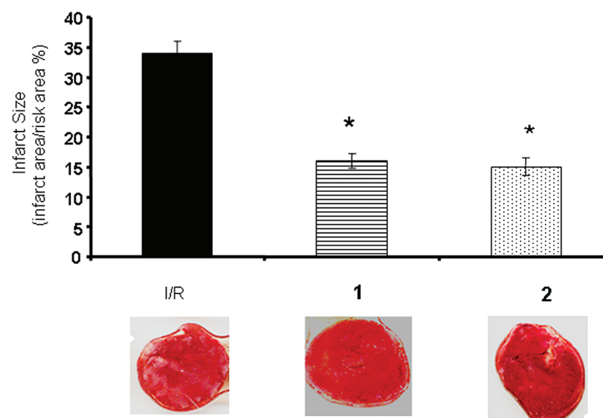

Figure 3. Effect of parrodienes on the infract size determined by TTC methods. Isolated hearts were perfused with KHB only or KHB + 50 mM parrodienes [octatrienal (**1**)/octatrienal K^+ salt (**2**)] for 15 min followed by 30 min of global ischemia and 120 min of reperfusion in the working mode. Results are expressed as the means ± SEM of six groups of heart/group. Comparisons were made to the values of test compound-free control group. $*p < 0.05$.

increased after ischemia reperfusion, but treatment with **1** and **2** reduced the activity of caspase 3 and prevented cardiomyosite apoptosis (Figure 5).

Effects 1 or 2 on the Generation of Survival Signal and HO-1 Protein. Having confirmed that **1** or **2** reduced myocardial ischemic injury, it was investigated whether these parrodienes could generate any survival signal. Since activation of Akt triggers the major signaling pathway toward cell survival, the activation of Akt

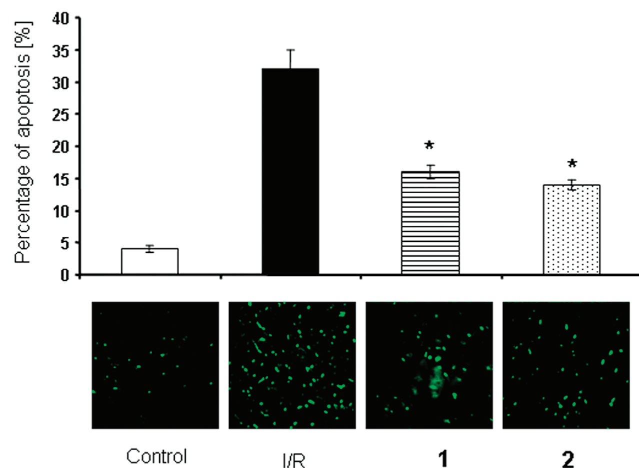

Figure 4. Effects of parrodienes **1** and **2** on cardiomyocyte apoptosis. Isolated hearts from control (nonischemic) rats were perfused with the KHB buffer without subjecting to ischemia and reperfusion. Isolated hearts from ischemic control rats [I/R] ($n = 6$) and treated rats ($n = 6$ from each group) were subjected to 15 min of perfusion with KHB only or KHB + parrodienes followed by 30 min of global ischemia and 2 h of reperfusion. Cardiomyocyte apoptosis was measured by Tdt-mediated dUTP nick end labeling with a Promega kit. Values are mean ± SEM; $*p < 0.05$ vs test compound free I/R. Representative photomicrographs are shown below the bar graphs.

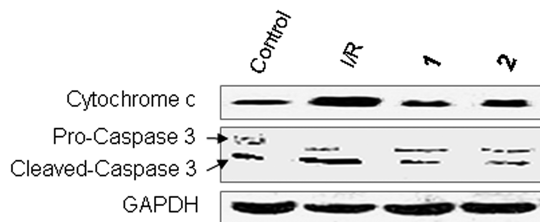

Figure 5. Western blot analysis of cytochrome *c* and caspase 3 in heart tissue obtained from control-BL, control-I/R, octatrienal (**1**), and octatrienoate K^+ salt (**2**) perfused heart. The isolated rat hearts were perfused for 15 min with only KHB buffer or KHB + **1**/**2**. The hearts were then subjected to 30 min of global ischemia followed by 2 h of reperfusion in the working mode. At the end of the experiment, the hearts were frozen in liquid nitrogen for subsequent Western blot analysis. GAPDH was used as a loading control. Figures are representative images of three different groups, and each experiment was repeated at least three times.

was determined by Western blotting. The effects of **1** and **2** on Bcl-2 and HO-1 proteins were also determined. Figure 6 shows that activation of Akt by phosphorylation at ser 473 was reduced after ischemia reperfusion in the case of the ischemic control group. However, **1** and **2** prevented the reduction of Akt phosphorylation due to ischemia reperfusion. The Bcl-2 and HO-1 proteins were also down-regulated after ischemia/reperfusion, but these parrodienes prevented this down-regulation.

Perfusing the heart with **1** and **2** resulted in improved postischemic ventricular function, reduced myocardial infarct size, reduced cardiomyosite apoptosis, increased level of anti-apoptotic Bcl-2 protein, and increased level of activated survival protein Akt.

Existing evidence indicates that the mitochondrial electron transport chain is a prime source of reactive oxygen species along with others such as xanthine oxidase, cytochrome P-450 enzymes, and NO synthase.[7] Also, the mitochondria are extremely susceptible to oxidative damage. Playing a key role in energy generation, this organelle is responsible for determining the longevity of cardiomyocytes. When there is an increased oxidative stress caused by ischemia reperfusion, this initiates apoptotic cell death.[8] For the

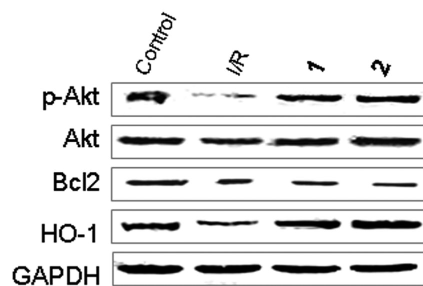

Figure 6. Western blot analysis of the p-Akt, Akt, Bcl-2, and HO-1 proteins in heart tissue obtained from control-BL, control-I/R, and octatrienal (**1**)/octatrienoate K^+ salt (**2**) perfused rats. The isolated rat hearts were perfused for 15 min with KHB buffer or KHB + **1**/**2**. The hearts were then subjected to 30 min of global ischemia followed by 2 h of reperfusion in the working mode. At the end of the experiment, the hearts were frozen in liquid nitrogen for Western blot analysis. GAPDH was used as a loading control. Figures are representative images of three different groups, and each experiment was repeated at least three times.

maintenance of mitochondrial integrity, membrane potential is likely to have an influence on myocardial energy production and the ultimate survival of the cells. Cellular injury is directly related to changes of mitochondrial architecture. Cytochrome *c* is an inner mitochondrial membrane protein and is released during cellular injury into the cytosol,[9] leading to the formation of an apoptotic complex, via the activation of a caspase cascade, which is initiated by caspase 9, leading to the activation and cleavage of procaspase 3 into two fragments.[10] The results of the present study showed the ischemia-induced release of significant amounts of cytochrome *c* into the cytosolic fraction and reduced the amount of procaspase 3 in the case of control hearts. This was reversed when hearts were perfused with parrodienes **1** and **2**. The amount of activated/cleaved caspase 3 was increased after ischemia in the case of control hearts, while perfusion with these parrodienes completely reversed the effect, indicating its ability to generate an antiapoptotic response. Consistent with these findings, it was also observed that the number of apoptotic cardiomyocytes was significantly reduced by parrodienes **1** and **2**.

Ischemia/reperfusion activates the death signal pathway at the expense of inhibition of the survival signal pathway, resulting in cardiomyocyte death.[11] We have examined several components of this survival pathway, which are known to be significantly affected by ischemia/reperfusion. Akt is the key regulator of PI-3-kinase-mediated cell survival, and constitutive activation of Akt by phosphorylation is sufficient to block cell death by a variety of apoptotic stimuli.[12] Akt activation has been shown to decrease apoptosis, as well as to reduce infarct size and to improve cardiac function after I/R.[13] Akt has been shown to inhibit cytochrome *c* release via Bcl-2 following an apoptotic stimulus.[14] Although the exact cardioprotective mechanism via Akt is still not completely known, it appears that Akt acts on multiple targets to provide its protective effects. Akt can also induce the antiapoptotic protein Bcl-2, which provides protection of mitochondria by acting on multiple targets.[15]

The results of the present study have shown that activation of Akt via phosphorylation and activation of Bcl-2 protein were reduced as a result of ischemia reperfusion in the case of the control heart, but in the case of the hearts perfused by **1** and **2** there was a significant induction in the expression of phospho-Akt and Bcl-2 proteins after ischemia, which is consistent with the results of a previous study.[16]

HO-1 is a cardioprotective protein and a phase 2 enzyme that becomes activated during the antideath signal. The effect of parrodienes **1** and **2** on the expression of the HO-1 protein was also determined. The results showed down-regulation of HO-1 after ischemia/reperfusion as expected in the case of the control heart,

and this down-regulation was prevented by **1** and **2**. The prevention of ischemia/reperfusion-mediated loss of HO-1 protein by **1** and **2** appears to play a role in cardioprotection.

Experimental Section

Chemicals. 2,4,6-Octatrienal (**1**) and 2,4,6-octatrienoate K^+ salt (**2**), synthesized following literature procedure,[2,17,18] were produced by the Institute of Organic Chemistry of the Faculty of Pharmacy of the University of Milan. These compounds were characterized by spectroscopic data analysis and were provided in 99% purity.

Animals. All animals used in this study received humane care in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. Sprague−Dawley male rats weighing 250−300 g were used. The rats were fed *ad libitum* regular rat chow with free access to water until the start of the experimental procedure. The rats were assigned randomly to one of three groups: group I control; group II 2,4,6-octatrienal (**1**); group III 2,4,6-octatrienoate K^+ salt (**2**). The treated rat hearts were perfused with either **1** or **2** at 50 mM in KHB buffer for 15 min prior to subjecting the heart to ischemia, while the control group was perfused with the buffer only.

Isolated Working Heart Preparation. Rats were anesthetized with sodium pentobarbital (80 mg/kg body weight, i.p.) (Abbott Laboratories, North Chicago, IL) and anticoagulated with heparin sodium (500 IU/kg body weight, i.v.) (Elkins-Sinn, Inc., Cherry Hill, NJ) injection. After ensuring sufficient depth of anesthesia, thoracotomy was performed, and then the hearts were perfused in the retrograde Langendorff mode at 37 °C at a constant perfusion pressure of 100 cm of water (10 kPa) for a 5 min washout period.[19] The perfusion buffer used in this study consisted of a modified Krebs-Henseleit bicarbonate buffer (KHB) (in mM: sodium chloride 118, potassium chloride 4.7, calcium chloride 1.7, sodium bicarbonate 25, potassium biphosphate 0.36, magnesium sulfate 1.2, and glucose 10). The Langendorff preparation was switched to the working mode following the washout period.

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 the hearts were perfused with either KHB only or KHB with either of the compounds (**1** and **2**) at a concentration of 50 mM, as mentioned above. This was followed by a 10 min washout with KHB buffer. The hearts were then subjected to 30 min of global ischemia followed by 2 h of reperfusion.[20] The first 10 min of reperfusion was in the retrograde mode to allow for postischemic stabilization and thereafter in the working mode, to allow for assessment of functional parameters, which were recorded at 30, 60, and 120 min reperfusion.

Assessment of Cardiac Function. Aortic pressure was measured using a Gould P23XL pressure transducer (Gould Instrument Systems Inc., Valley View, OH) connected to a sidearm of the aortic cannula, and the signal was amplified using a Gould 6600 series signal conditioner and monitored on a CORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA).[21] The heart rate (HR), left ventricular developed pressure (LVDP) (defined as the difference of the maximum systolic and diastolic pressures), and the first derivative of developed pressure (dp/dt) were all derived or calculated from the continuously generated pressure signal. Aortic flow (AF) was measured using a calibrated flow-meter (Gilmont Instrument, Inc., Barrington, IL), while coronary flow (CF) was measured by timed collection of the coronary effluent dripping from the heart.

Infarct Size Estimation. At the end of reperfusion, a 1% (w/v) solution of triphenyl tetrazolium chloride in phosphate buffer was infused into an aortic cannula for 20 min at 37 °C.[21] The hearts were excised and stored at −70 °C. Sections (0.8 mm) of each frozen heart were fixed in 2% paraformaldehyde, placed between two coverslips, and imaged digitally using a Microtek Scan Maker 600z. To quantitate the areas of interest in pixels, NIH image 5.1 (a public-domain software package) was used. The infarct size was quantified and expressed in pixels.

TUNEL Assay for the Assessment of Apoptotic Cell Death. Immunohistochemical detection of apoptotic cells was carried out using a Dead-End Fluorometric TUNEL[22] system (Promega, Madison, WI). The heart tissues were immediately put in 10% formalin and fixed in an automatic tissue-fixing machine. The tissues were carefully embedded in the molten paraffin in metallic blocks, covered with flexible plastic molds, and kept under freezing plates to allow the paraffin to solidify. The metallic containers were removed and the tissues became embedded in paraffin on the plastic molds. Prior to analyzing tissues for apoptosis, paraffin was removed from tissue sections with xylene and washed in succession with different concentrations of ethanol (absolute, 95%, 80%, 70%). Then, the TUNEL staining was done according to the manufacturer's instructions. The fluorescence staining was viewed with a fluorescence microscope (AXIOPLAN2 IMAGING) (Carl Zeiss Microimaging, Inc., Thornwood, NY). The number of apoptotic cells was counted and expressed as a percent of total myocyte population.

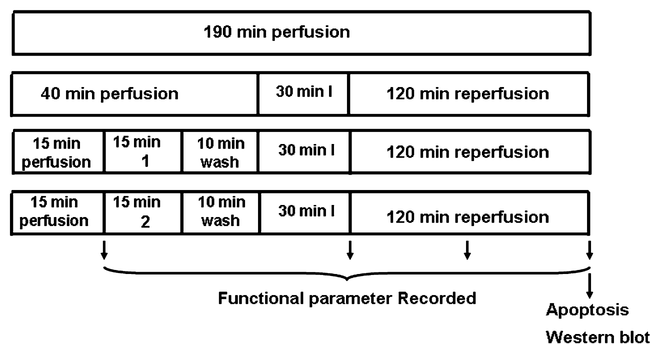

Figure 7. Schematic representation of perfusion protocol of different groups of hearts.

Preparation of Cytosolic Extracts. Tissues (frozen in liquid nitrogen and stored at −80 °C) were homogenized in 1 mL of buffer A (25 mM Tris-HCl, pH 8, 25 mM NaCl, 1 mM Na-orthovanadate, 10 mM NaF, 10 mM Na pyrophosphate, 10 nM okadaic acid, 0.5 mM EDTA, 1 mM PMSF, and 1x Protease inhibitor cocktail) in a Polytron homogenizer. Homogenates were centrifuged at 2000 rpm at 4 °C for 10 min. Supernatant from the above centrifugation was further centrifuged at 10 000 rpm at 4 °C for 20 min, and the resultant supernatant was used as cytosolic extract. Cytosolic extracts were aliquoted, snap frozen, and stored at −80 °C until used. Total protein concentration in the cytosolic extract was determined using a BCA protein assay kit (Pierce, Rockford, IL).

Western Blot Analysis. Proteins from the left ventricular biopsies were separated by SDS-PAGE and transferred to nitrocellulose filters.[23] Filters were blocked in 5% nonfat dry milk and probed with primary antibody overnight at 4 °C. Primary antibodies such as Akt, phospho-Akt (Ser 473), hemeoxygenase-1, and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); Bcl-2, caspase-3, and cytochrome *c* were obtained from Cell Signaling Technology (Beverly, MA). All primary antibodies were used at a dilution of 1:1000. Protein bands were identified with horseradish peroxidase conjugated secondary antibody (1:2000 dilution) and Western Blotting luminol reagent (Santa Cruz Biotechnology). GAPDH was used as loading control. The resulting blots were digitized, subjected to densitometric scanning using a standard NIH image program, and normalized against loading control.

Statistical Analysis. The values for myocardial functional parameters, total and infarct volumes and infarct sizes, and cardiomyocyte apoptosis were all expressed as the mean ± standard error of mean (SEM). Analysis of variance test followed by Bonferoni's correction was first carried out to test for any differences between the mean values of all groups. If differences between groups were established, the values of the treated groups were compared with those of the control group by a modified t test. The results were considered significant if $p < 0.05$.

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