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Cardiomyocytic Apoptosis Limited by Bradykinin *via* Restoration of Nitric Oxide after Cardioplegic Arrest

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Background. **Our previous studies revealed that cardioplegia-induced cardiac arrest under cardiopulmonary bypass (CPB) decreased cardiomyocytic nitric oxide and increased apoptosis. We hypothesized that pretreatment with bradykinin (BK) would improve the profile of anti-apoptotic proteins and inhibit cardiomyocytic apoptosis.**

Materials and Methods. **New Zealand white rabbits received total CPB. Rabbits were weaned from CPB and reperfused for 4 h. Blood was sampled at various time points. Bradykinin and/or nitric oxide synthase (NOS) inhibitors or BK-receptor antagonists were infused systemically 30 min before beginning of CPB, and continued throughout the procedure. The ascending aorta was cross-clamped for 60 min while cold crystalloid cardioplegic solution was intermittently infused into the aortic root. The hearts were harvested and studied for evidence of apoptosis and ischemia/reperfusion induced inflammation-related cytokine production by cardiomyocytes.**

Results. **Our results revealed that bradykinin supplementation during cardioplegia could prevent I/R-induced inflammatory and apoptotic effects, which could be reversed with a NOS inhibitor. BK antagonists and NOS inhibitors worsened the inflammatory and apoptotic responses of cardiomyocytes, which could be reversed with an exogenous NO donor.**

Conclusions. **Restoring the NO concentration after cardioplegia-induced cardiac arrest (CCA) under CPB with bradykinin could modulate (1) the nuclear translocation of NF-κB, (2) the plasma levels of inflammation-related cytokines, (3) the Bcl-2/Bax ratio, and (4) the occurrence of apoptosis. Exogenous bradykinin administration was associated with the myocardial apoptotic response by inhibition of NF-κB translocation, inflammatory cytokine production, Akt activation, and elevation of the Bcl-2/Bax ratio *via* a NO-mediated pathway.**

Key Words: **apoptosis; bradykinin; cardioplegia; ischemia; nitric oxide.**

INTRODUCTION

In the setting of cardiac global ischemia/reperfusion (I/R) insult during cardiopulmonary bypass (CPB) with cardioplegia-induced cardiac arrest (CCA), changes in vascular permeability, in endothelial integrity, and in cardiomyocytic damage are consequences of numerous simultaneous stimuli, including the release of endogenous vasoconstrictors, such as endothelin and angiotensin II, and the activation of platelets and leukocytes to form cellular aggregates capable of occluding vessels within the microcirculation [1]. These pathologic processes are mediated in part by a reduction in the release of nitric oxide (NO) from endothelial NO synthase, which has been shown to occur after ischemic arrest [2]. NO synthesis may be impaired by depletion of endogenous L-arginine, the natural precursor of NO [3], or possibly by blocking the recycling of L-citrulline to L-arginine [4].

Apoptosis and necrosis are two different types of cell death. Apoptosis has been considered to be one of the mechanisms of cell loss during I/R injury [5]. Although the mechanism responsible for I/R-induced apoptosis is incompletely understood, it has been demonstrated that I/R-induced apoptosis is regulated mainly by the

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Bcl-2 family of proteins and the cysteine protease family of caspases [5–7]. It has been shown that the transcriptional factor NF-κB can be induced by a multitude of stimuli, including cytokines and reactive oxygen species. Degradation of IκB results in NF-κB activation, which controls the transcription of several genes involved in inflammatory responses, cell growth, survival and apoptosis [8]. Our previous study revealed that CCA-induced cardiomyocytic I/R injury under CPB could result in NF-κB activation with nuclear translocation, which up-regulated pro-inflammatory cytokines production, increased caspase activity, and induced cardiomyocytic apoptosis [7, 9].

Several cell-survival factors have been shown to improve the profile of anti-apoptotic proteins and prevent myocardial apoptosis [6]. Bradykinin (BK) has been widely accepted as one of the endogenous protective factors to improve left ventricular performance, attenuate myocardial necrosis, and reduce the incidence of cardiomyocytic apoptosis after CCA-induced I/R injury under CPB [10]. Kono *et al.* reported that bradykinin could activate NO synthase and subsequently increase nitric oxide production [11]. However, it is not known if BK affects I/R-induced apoptosis-related proteins and myocardial apoptosis *via* NO-related pathways. Therefore, we hypothesized that BK would improve the profile of anti-apoptotic proteins, and prevent apoptosis after CCA-induced I/R injury under CPB *via* a NO-mediated pathway.

MATERIALS AND METHODS

Animal Model of Global Cardiac Ischemia-Reperfusion Injury with Cardiopulmonary Bypass

Animals were housed individually and were provided laboratory chow and water *ad libitum*. All experiments were approved by the Chang Gung Memorial Hospital Animal Care and Use Committee, and conformed to the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health (NIH publication no. 85-23, 1996). The methods for anesthesia, surgery, instrumentation of the heart, and physiologic monitoring have been described previously [9].

Ten male New Zealand white rabbits in each group, (2.5–3.5 kg each) were premedicated with ketamine (10 mg/kg intramuscularly), anesthetized with sodium pentobarbital (30 mg/kg intravenous injection), and then given intermittent boluses of pentobarbital (5 mg/kg) and diazepam (2 mg) as needed during the experiment. Each rabbit was intubated with an endotracheal tube, and was ventilated with oxygen-enriched room air using a respirator. The left femoral artery was catheterized for collection of hemodynamic data and the left femoral vein was catheterized for the administration of fluids. Physiologic variables, including heart rate, blood pressure, and maximal slope of systolic pressure increment (dP/dT) were analyzed using a computer equipped with an analog/digital converter (DASA 4600; Gould, Inc., Valley View, Ohio) and software (BioCybernatics 1.0; Taipei, Taiwan). Rectal temperature was also monitored.

After a median sternotomy, the pericardium was opened and tented to cradle the heart. The aorta was cannulated for aortic perfusion after intravenous heparinization (250 U/kg). The right atrium was cannulated for venous return. The left ventricle was vented directly by a catheter inserted though the left atrial appendage. CPB was instituted using a membranous oxygenator (Polystan Neonatal Oxygenation System; Polystan, Inc. Vaerlose, Denmark) with a flow rate of 40 mL/kg/min. After stabilization, the ascending aorta was cross-clamped, except in group 1. An internal mammary artery cannula (0.5 mm; Medtronic DLP, Grand Rapids, MI) was inserted for antegrade delivery of cardioplegic solution and simultaneous measurement of infusion pressure. All infusions of cardioplegic solution were administered at a pressure of 20 to 30 mmHg. The body temperature during CPB was kept around 28°C. Cold (4°C) crystalloid cardioplegic solution (4 mL/kg; Plegisol, Abbott Laboratories, North Chicago, IL) was infused into the aortic root every 20 min. Initial induction of cardiac arrest was accomplished using 20 Meq/L potassium crystalloid cardioplegic solution, and was followed by intermittent infusion of 8 Meq/L potassium crystalloid cardioplegic solution.

Extracorporeal circulation was established with sternotomy in all seven groups. The ascending aorta was cross-clamped for 60 min. Bradykinin (10^{-5} M; BK), bradykinin receptor antagonist (10^{-9} M; HOE140), non-selective NO synthase inhibitor (N-omega-nitro-L-arginine methyl ester, 10 mg/kg; L-NAME), inducible NO synthase (iNOS) inhibitor (aminoguanidine, 150 mg/kg; AG), or exogenous NO donor (S-nitrosothiols, 10 μM/L/min; GSNO) was added to the cardioplegic solution. The doses of various drugs applied in the present studies were comparable with the doses used in previous *in vivo* cardiac ischemia-reperfusion and pharmacokinetic studies [7, 10, 11].

After CPB was established with sternotomy, the cardioplegic solution was supplemented with the different test drugs according to the following protocol:

Group 1: Cold crystalloid cardioplegic solution was not infused, nor was cross-clamping performed.

Group 2: Crystalloid cardioplegic solution without other drugs was infused every 20 min after cross-clamping *via* the aortic root.

Group 3: Cold crystalloid cardioplegic solution supplemented with BK was infused every 20 min after cross-clamping *via* the aortic root.

Group 4: Cold crystalloid cardioplegic solution supplemented with BK and L-NAME was infused every 20 min after cross-clamping *via* the aortic root.

Group 5: Cold crystalloid cardioplegic solution supplemented with BK and AG was infused every 20 min after cross-clamping *via* the aortic root.

Group 6: Cold crystalloid cardioplegic solution supplemented with BK and HOE140 was infused every 20 min after cross-clamping *via* the aortic root.

Group 7: Cold crystalloid cardioplegic solution supplemented with BK, HOE140, and GSNO was infused every 20 min after cross-clamping *via* the aortic root.

After 60 min of aortic cross-clamping, the cross-clamp was removed, and the rabbit was rewarmed to a body temperature of 37°C. CPB was discontinued, and the mean arterial pressure was kept around 40 mm Hg. The hearts were reperfused for another 4 h in the working state before excision. Blood samples were obtained before surgery and serially after surgery for measurement of cardiac troponin I (cTnI), cytokines, and adhesion molecules. Blood samples were taken at the following time points: baseline (immediately after anesthesia induction), upon establishment of CPB, and 4 h after reperfusion. Platelet-poor plasma was prepared by centrifugation at 2000 $\times g$ for 15 min. The plasma was stored in polypropylene tubes at –70 °C until use.

Tissue Preparation

The heart was removed rapidly after 4 h reperfusion. The myocardium was isolated from the reperfused hearts. Tissue specimens were fixed in 10% neutral buffered paraformalin, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. The specimens were cut into 6-mm-thick slices for microscopic examination. The remaining tissue samples were placed in liquid nitrogen for further tissue survey.