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The Nonselective β-Blocker Carvedilol Suppresses Apoptosis in Human Cardiac Tissue: A Pilot Study

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ABSTRACT

Background: Cardioplegia and reperfusion of the myocardium may be associated with cardiomyocyte apoptosis and subsequent myocardial injury. To establish a pharmacologic strategy for the prevention of these events, this study aimed to verify the reliability of our human cardiac model and to evaluate the antiapoptotic properties of the nonselective β-blocker carvedilol during simulated cardioplegia and reperfusion ex vivo.

Methods: Cardiac biopsies were retrieved before induction of cardiopulmonary bypass from the auricle of the right atrium of patients undergoing elective coronary artery bypass grafting. Biopsies were exposed to ex vivo conditions of varying periods of cardioplegia/reperfusion (30/10 minutes, 60/20 minutes, 120/40 minutes). Group I was the untreated control (n = 15), group II was the treated control (cardioplegia/reperfusion, n = 15), and group III was the experimental group (cardioplegia/reperfusion plus carvedilol, n = 15). Immunostaining for antibodies to activated caspase 3 and poly(ADP-ribose) polymerase 1 (PARP-1) cleavage was used to detect apoptosis.

Results: The percentage of apoptotic cardiomyocytes was significantly lower ($P < .05$) in group I than in group II, revealing a time-dependent increase. In group III, carvedilol treatment suppressed apoptosis significantly ($P < .05$).

Conclusion: Carvedilol significantly suppresses apoptosis in our ex vivo setting. This finding warrants further studies to evaluate the potential beneficial effects of carvedilol in suppressing ischemia/reperfusion injury in clinical settings.

INTRODUCTION

In cardiac surgery, cardioplegia and reperfusion of the myocardium are used in many procedures when a temporarily arrested myocardium is required; however, apoptosis of cardiomyocytes may occur because of the exposure to cardioplegia and reperfusion [Bai 2005]. Apoptosis is the ultimate result of multiple convergent signaling pathways, which are triggered by such events as nutrient and oxygen deprivation, intracellular calcium overload, and excessive production of reactive oxygen species [Bai 2005]. In the setting of cardiac surgery, these events can finally cause contractile dysfunction of the myocardium [Murriel 2004] and atrial fibrillation [Ak 2005]. Apoptosis of cardiac nonmyocytes also contributes to maladaptive remodeling and a transition to decompensated congestive heart failure [Khoynezhad 2007]. In recognition of this potential impact of apoptosis on clinical outcomes, there is a demand for pharmacologic strategies. Pharmacologic blockade of β-adrenoreceptors has been shown in an animal model to reduce apoptosis during extracorporeal circulation [Zhang 2003].

Our pilot study was performed to evaluate our presented human cardiac model during simulated cardioplegia and reperfusion ex vivo with respect to the end points of feasibility and reliability. In addition, we conducted this study to investigate the effect of carvedilol on the end point of apoptosis reduction in cardiomyocytes. The results should clarify whether the addition of carvedilol during cardioplegia and reperfusion can produce any potential clinical benefit.

The aim of this study was to evaluate the effect of the nonselective β-blocker carvedilol on apoptotic events in human myocardium during ex vivo simulation of cardioplegia and reperfusion, an approach that, to our knowledge, has not yet been described.

MATERIALS AND METHODS

Ethics Declaration

The investigation conforms to the principles outlined in the Declaration of Helsinki. Approval was granted by the ethics committee of the Faculty of Medicine of the Eberhard Karls University, Tübingen, Germany (approval reference no. 40/2007V).

Patient Characteristics

The study protocol was approved by the ethics committee of the Faculty of Medicine of the Eberhard Karls University Tübingen. We included in this study 15 patients who were to

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undergo elective coronary artery bypass grafting surgery, and the patients provided informed consent for study participation. The mean patient age was 63 years (range, 50-70 years), and 55% of the patients were female. The mean body mass index was 26 kg/m^2 (range, 23-30 kg/m^2). The mean left ventricular ejection fraction was 61% (range, 55%-70%). The mean number of diseased coronary vessels was 3 (range, 2-3). The mean number of infarctions in the patients' history was 1 (range, 1-3). The patients' basic medications consisted of β-blockers (47.5 mg Beloc Zok™ twice per day), angiotensin-converting enzyme inhibitors, statins, and diuretics. All patients had a sinus rhythm.

Materials

Tissue was retrieved before cardiopulmonary bypass from the auricle of the right atrium of the patients and was processed immediately. Each biopsy was transmurally divided into 13 pieces (0.5-1 cm^2), which were placed separately in microperfusion chambers with continuous perfusion. Cardiac samples were outside the body for a maximum of 30 minutes before being mounted and tested in the chamber system, but the oxygen supply was maintained continuously during this period by bubble-oxygenation of the Krebs-Henseleit buffer in the petri dish (Greiner Bio-One, Frickenhausen, Germany).

Chemicals and Buffer Solutions

We used a modified Krebs-Henseleit buffer, which consisted of 115mM NaCl, 4.5mM KCl, 1.18mM $MgCl_2$, 1.25mM $CaCl_2$, 1.23mM NaH_2PO_4, 1.19mM Na_2SO_4, 80mM glucose, and 10mM HEPES. The pH was adjusted to 7.4 at 37°C with NaOH.

Cardioplegic Solution. We first prepared calcium-free Krebs-Henseleit buffer containing EGTA (115mM NaCl, 4.5mM KCl, 1.18mM $MgCl_2$, 0.5mM EGTA, 1.23mM NaH_2PO_4, 1.19mM Na_2SO_4, 80mM glucose, and 10mM HEPES; pH adjusted to 7.4 at 37°C with NaOH). We then added 1 part of a solution containing 20mM Tris, 60 mmol K^+, and anionic polypeptides to the isoionic point to 4 parts of the calcium-free Krebs-Henseleit buffer. This solution served as the cardioplegic solution and was administered at 4°C, in analogy to our clinical regimen. The resulting K^+ concentration in this mixture was 16.5mM.

Carvedilol. Carvedilol, 1-[carbazol-4-yloxy]-3-[[(2-(o-methoxyphenoxy)ethyl]amino]-2-propanol, was originally introduced as a nonselective β-adrenoreceptor antagonist and is used in current clinical settings for the treatment of hypertension and heart failure. The range of therapeutic concentrations used in clinical settings is 3.5μM to 20μM [Lotze 2002; Wang 2006]. The carvedilol concentration used in this study was 10μM, similar to that used in previous experimental settings [Wang 2006].

Cell Viability

The viability of cardiomyocytes in tissue samples was assessed by trypan blue exclusion before each experiment. Only samples consisting of ≥99% viable cardiomyocytes were processed further in the experiments of this study.

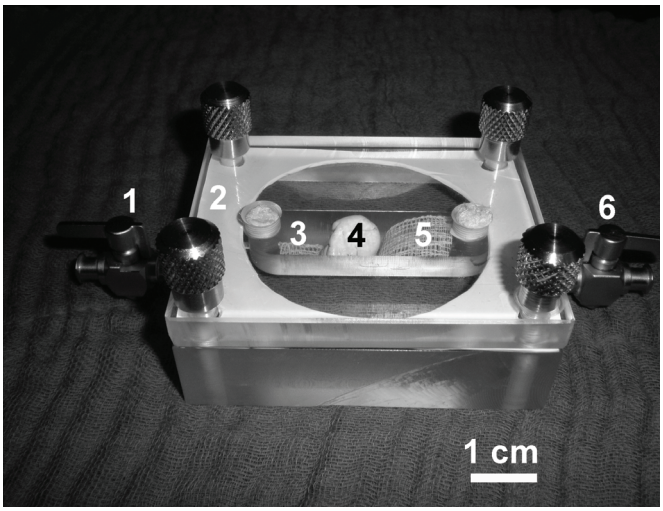

Figure 1. Microperfusion chamber. The perfusate enters the Plexiglas chamber (2), proceeds through the pipe (1), and fills the rectangular chamber (3). Once laminar flow is constituted, the cardiac tissue (4) is physically fixed to the nylon net (5), which spans a 135° angle. The fluid exits on the opposite side (6). A rubber layer is placed between the bottom and upper parts of the chamber for sealing, and the chamber is fastened with 4 screws.

Microperfusion Chamber

Our self-developed, previously described microperfusion chamber [Weinlich 2002] was modified to investigate larger samples. It consisted of 2 components (Figure 1). The first component is a temperature-controlled Plexiglas block containing a rectangular cavity that forms a chamber with the following dimensions: 5.5 cm long × 1.5 cm wide × 1.25 cm high). The second component, which was mounted over the first, consists of another Plexiglas block that forms the ceiling of the chamber. A nylon net with a pore size of 400 μm is mounted diagonally in this chamber. Perfusion is enabled in the chamber by the introduction of a thin pipe at one end of the Plexiglas chamber and its exit at the other end. A thin rubber layer between each component seals the microperfusion chamber. The biopsy sample is fixed physically at the nylon net by the laminar flow (perfusion velocity, 5 mL/min) of the hydrostatic system of perfusion through the chamber.

Experimental Groups

The protocol was designed to simulate routine clinical procedures of administering cardioplegic solution at the same K^+ concentration (16.5mM) and temperature (4°C). We used the following 3 groups in the experiments: group I, the untreated control (n = 15); group II (treated control), which consisted of cardioplegia and reperfusion without carvedilol (n = 15); and group III, which consisted of cardioplegia and reperfusion with carvedilol (n = 15). In group III, cardiomyocytes were treated continuously with 10μM carvedilol. In general, each assay was carried out with the samples from 1 patient (ie, each patient's samples were analyzed separately).

Ischemia/Reperfusion Assay

The cardiac samples in the microperfusion chambers were initially equilibrated with Krebs-Henseleit buffer for 5 minutes (32°C and continuously bubble-oxygenated with carbogen (95% O_2 and 5% CO_2) to attain a PO_2 of 25 to 30 kPa and a pH of 7.4. Then, the cardioplegic solution (4°C) was administered for 5 minutes. To induce ischemic injury during the cardioplegia period, we stopped the perfusion of the microperfusion chamber and discontinued the oxygen supply. The cardiac samples were subjected to various periods of cardioplegia (30, 60, or 120 minutes) followed by reperfusion for one third of the chosen cardioplegia time (ie, 10, 20, or 40 min), as in our surgical routine. For reperfusion, we used Krebs-Henseleit buffer at 35°C. Finally, the cardiac samples were snap-frozen in liquid nitrogen.

Immunohistochemical Detection of Apoptosis

The slides with 10-µm cryosections of the samples were processed before staining according to the recommendations of the manufacturer (Epitomics, Burlingame, CA, USA). The described chemicals were purchased from Biochrom (Berlin, Germany). In brief, the cryosections were immersed in the staining dish containing the antigen retrieval solution, which was prepared by adding 9 mL of 0.1M citric acid and 41 mL of 0.1M sodium citrate to 450 mL distilled H_2O and adjusting to pH 6.0. After warming of the slides for 30 minutes in a rice cooker and cooling, the slides were washed with TBST (Tris-buffered saline and 0.1% Tween 20) for 5 minutes on a shaker. Endogenous peroxidases were inactivated by covering the slides with 3% hydrogen peroxide for 10 minutes and then washing with TBST. The slides were then immersed for 1 hour in the blocking solution, which consisted of phosphate-buffered saline (Dulbecco phosphate-buffered salts) and 10% bovine serum albumin.

The cryosections were incubated overnight in a humidified chamber (4°C) with antibodies to poly(ADP-ribose) polymerase 1 (PARP-1) cleavage (Epitomics). PARP-1, a zinc-dependent DNA-binding protein that recognizes DNA strand breaks, is presumed to play a role in DNA repair. PARP-1 is cleaved in vivo by caspase 3 [Tewari 1995]. The antibody recognizes only the p25 cleaved form of PARP-1.

In addition, cryosections were stained with antibodies to activated caspase 3 (Epitomics). Caspases are a family of cytosolic aspartate-specific cysteine proteases involved in the initiation and execution of apoptosis. Caspase 3 (also known as apopain, SCA-1, Yama, and CPP32) is a member of the apoptosis-execution functional group of caspases and is either partially or totally responsible for the proteolytic cleavage of many key proteins during apoptosis. Caspase 3 is a cytosolic protein that is found in cells as the inactive 35-kDa proenzyme. It is activated by proteolytic cleavage into 2 active subunits only when cells undergo apoptosis [Ak 2005].

Caspase 3 was then detected by diluting horseradish peroxidase–conjugated antirabbit antibody in the blocking solution according to the recommendation of the manufacturer (Epitomics), applying the antibody preparation to each section, and incubating for 1 hour at room temperature.

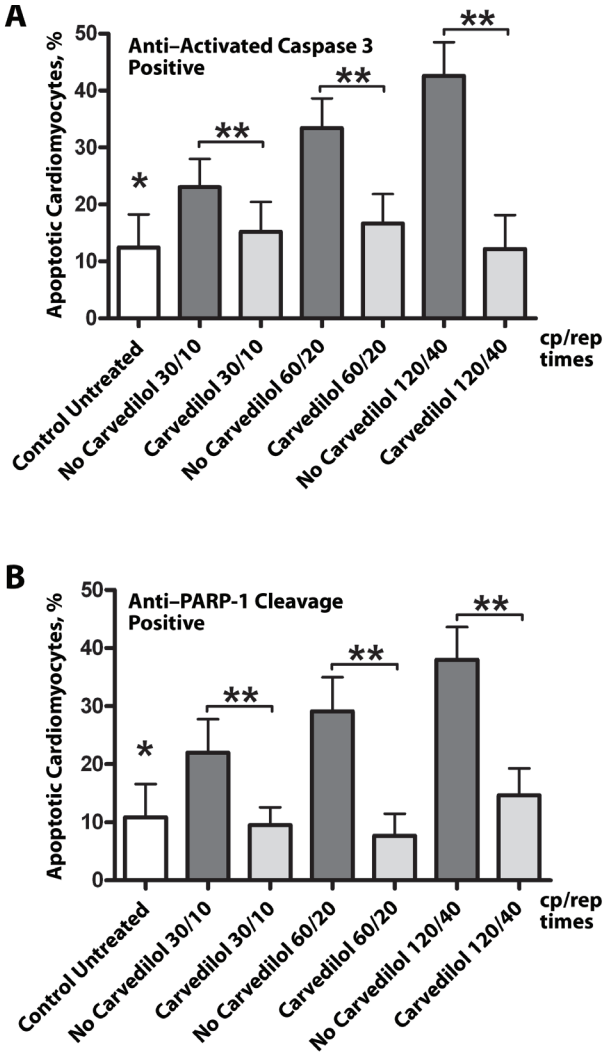

Figure 2. Percentage of cardiomyocytes positive for antibodies to activated caspase 3 (A) and antibodies to PARP-1 cleavage (B). The treated control group (No Carvedilol) shows a significant time-dependent increase in apoptotic cardiomyocytes, compared with the untreated control group (*P < .05) and the cardiomyocytes treated with carvedilol (**P < .05). Carvedilol reduces apoptosis significantly (P < .05), even with increasing durations of cardioplegia and reperfusion. Results represent the mean ± SD of the combined results of 15 independent assays. cp/rep times indicates the cardioplegia/reperfusion times in minutes.

Statistical Analysis

Analysis of calcium recordings and graphics were obtained with SigmaPlot software (version 9.0; SPSS, Chicago, IL, USA). Data are expressed as the mean ± SD. Statistical analysis was performed with GraphPad Prism software (version 5.0; GraphPad Software, La Jolla, CA, USA). Comparisons of groups were performed with repeated-measures 1-way analysis of variance, followed by the Tukey HSD post hoc test. A P value <.05 was considered statistically significant.

RESULTS

Immunohistochemical Detection of Apoptosis

Anti–Activated Caspase 3. Cardiomyocytes in the untreated group (group I) had a significantly lower percentage of apoptotic cells (13% ± 6%; $P < .05$) than the treated control group (group II) (Figure 2A), and the carvedilol treatment group (group III) had a significantly lower percentage of apoptotic cells than group II ($P < .05$; Figure 2A).

PARP-1 Cleavage. Cardiomyocytes in the untreated group (group I) had a significantly lower percentage of apoptotic cells (11% ± 6%; $P < .05$) than the treated control group (group II) (Figure 2B), and the carvedilol treatment group (group III) had a significantly lower percentage of apoptotic cells than group II ($P < .05$; Figure 2B).

DISCUSSION

Apoptosis Research

The simulation of ischemia in isolated cardiomyocyte models can provide important insights into the pathophysiology of myocardial ischemic injury and its underlying molecular mechanisms. Such evaluations include the analysis of major mediators of postischemic reperfusion injury, such as reactive oxygen species, dysregulation of intracellular Ca^{2+} overload, and neutrophil accumulation [Groholm 2004]. Apoptosis is an important mechanism of cellular death and is distinct from necrosis. Apoptosis has been implicated in the pathogenesis of a variety of degenerative and ischemic diseases [Communal 2002]. The members of the caspase family are key mediators of apoptosis. An extrinsic pathway involving death receptors on the cell surface leads to the activation of procaspase 8 and procaspase 3, which is followed by proteolysis of cellular substrates and consequent cell death. In an intrinsic pathway, intracellular and extracellular death signals are transmitted to mitochondria through members of the Bcl-2 family [Kubasiak 2002]. Several intracellular stimuli, including oxidative stress, are involved in the translocation of Bax and/or Bak to the mitochondria, which leads to dysfunction of this organelle, the release of proapoptotic proteins (such as cytochrome c and Smac), and the activation of caspase 9 [Danial 2004]. The extrinsic and intrinsic pathways are interconnected via Bid. Another important pathway that can lead to apoptosis is the endoplasmic reticulum (ER) pathway. Stress to the ER can also produce apoptosis. Caspase 12, an ER membrane–bound caspase, has been demonstrated to be a major mediator of ER stress-mediated apoptosis. Caspase 12 release from the ER is followed by the processing of downstream caspases, such as caspase 3, 7, and 9 [Rao 2001]. Most of these studies were performed on isolated mammalian cardiomyocytes [Kaiser 2004] or on isolated papillary muscle preparations [Ren 2002]. Obtaining human papillary muscle before cardioplegia, however, is not a realistic and clinically feasible option. Because we intended in this study to simulate the effect of cardioplegia/reperfusion on intact human cardiac tissue, samples were taken from the auricle of the right atrium and then subjected to cardioplegia in vitro in the microperfusion chamber. Our results indicate that the cardioplegia and reperfusion stimulus is adequate to induce apoptosis. This finding is comparable with other recent studies described in the literature, which have also demonstrated that ischemia and reperfusion trigger apoptosis [Miyamoto 2005].

Apoptosis Inhibition

Previous studies have shown that carvedilol blocks adrenoreceptor transmission of intracellular apoptosis-inducing signals. This result indicates that cardiomyocyte apoptosis is selectively mediated by β1-adrenoceptors [Zaugg 2000]. We found that 10μM carvedilol suppressed apoptosis in all experiments in this study. Caspase 3 activation and the resulting PARP-1 cleavage were lower with carvedilol treatment. The partial inhibition of apoptosis by carvedilol observed in our study also has been described in an animal model of end-stage heart failure [Kawai 2004]. Another reason for the partial suppression of apoptosis in cardiomyocytes could also be dose related, as described in previous studies [Wang 2005]. Carvedilol treatment inhibited apoptosis in a way such that a longer duration of cardioplegia and reperfusion did not increase the apoptosis rate significantly, whereas the apoptosis rate increased when carvedilol was absent. The high apoptosis rate in the control group, especially after 120 minutes of cardioplegia and 40 minutes of reperfusion, should be extrapolated to the in vivo situation with caution, because atrial myocardium and ventricular myocardium possess specific characteristics that may influence their susceptibility to ischemia/reperfusion injury. One such characteristic is the reported difference between atrial and ventricular myocardium in the distribution of potassium channels [Amos 1996], which contributes to the characteristic differences between atrial and ventricular action potentials and may lead to different responses to cardioplegia/reperfusion.

Study Limitations

The present study has a few potential limitations. First, clinical ischemia may be quite different from the simulated ischemia in our study. Unfortunately, there is currently no accepted standard for a clinically relevant "simulated ischemic exposure" for cells. Simulating the ischemic environment of the extracellular fluid that bathes the cells is quite complex because there are changes in many factors, and simulating all of these events is not currently possible. Therefore, although our use of a simulated ischemia is not perfect, we believe it does re-create a number of the important components of clinical ischemia. In addition, we used only a single carvedilol concentration in this study, but our study was analogous to previous studies in which a pharmacologically relevant concentration was used [Lotze 2002; Wang 2006]. Therefore, we did not investigate the detailed dose-response relationships of carvedilol with apoptotic events. Nevertheless, we were able to demonstrate with the carvedilol concentration used in this study that apoptotic events could be inhibited considerably. Furthermore, our primary purpose in this study was to test carvedilol's effect on apoptotic events in cardiomyocytes in this new experimental setting, rather than to study dose-response relationships. Our next step would be to verify our current

findings in an animal model. Our results, however, indicate a definite beneficial effect of carvedilol on apoptotic events.

CONCLUSIONS

The addition of carvedilol to the treatment of human cardiomyocytes in an ex vivo experimental setting with simulated cardioplegia and reperfusion produced a considerable reduction in apoptotic events. These findings warrant further studies to evaluate the potentially beneficial effects of β-blockade in the in vivo setting of cardioplegia as used in cardiac surgery.

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