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Modulation of the Nitric Oxide Metabolism Overcomes the Unresponsiveness of the Diabetic Human Myocardium to Protection Against Ischemic Injury

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Background. **We have demonstrated that diabetic human myocardium cannot be protected by ischemic preconditioning (IP) and identified a dysfunction of the mitochondria as the cause of the defect. Here we have investigated whether modulation of the nitric oxide (NO) metabolism can overcome the unresponsiveness of the diabetic myocardium to cardioprotection.**

Methods. **Myocardial slices (30–40 mg) obtained from the right atrial appendage of patients with diabetes undergoing elective cardiac surgery were randomized to the following protocol (n = 6/group): NO donor SNAP (100 μM), nonselective nitric oxide synthase (NOS) inhibitor L-NAME (100 μM), and selective neuronal NOS (nNOS) inhibitor TRIM (100 μM) for 20 min prior to 90 min ischemia followed by 120 min reoxygenation (37°C). Some preparations were subjected to ischemic/reoxygenation alone or to IP (5 min ischemia/5 min reoxygenation) to act as control. Tissue injury was assessed by creatine kinase (CK) released (IU/mg wet wt), and cell necrosis and apoptosis by propidium iodide and TUNEL (% of aerobic control).**

Results. **IP did not decrease CK release, cell necrosis or apoptosis in diabetic myocardium. However, NO donor SNAP, the nonspecific NOS inhibitor L-NAME, and the specific nNOS inhibitor TRIM significantly reduced CK leakage, cell necrosis, and apoptosis in diabetic myocardium.**

Conclusions. **These results demonstrate that both the provision of exogenous NO and the suppression of endogenous NO production result in potent protection of diabetic human myocardium overcoming the unresponsiveness of these tissues to cardioprotective therapies.**

Key Words: **ischemic injury; diabetes; cardioprotection; nitric oxide; nitric oxide synthase.**

INTRODUCTION

Diabetes is a multiple organ disease that also increases the mortality and morbidity in patients with coronary artery diseases [1]. It is also recognized that patients with diabetes undergoing coronary artery bypass graft have greater postoperative mortality and morbidity than patients without diabetes [2] but the reason for it remains unclear. We have previously observed greater oxidative stress induced by cardiopulmonary bypass in tissue from diabetic patients compared with tissue from nondiabetic patients [3]. This may explain the greater surgical risk for diabetic patients. We have also demonstrated that the diabetic human myocardium is unresponsive to protective interventions such as ischemic (IP) and pharmacologic preconditioning [4], and that dysfunctional mitochondria, probably the mitoK$_{ATP}$ channel, is responsible for the deficit [5]. We [6, 7] and other investigators [8, 9] have demonstrated that mitoK$_{ATP}$ channels are a key factor for eliciting IP and that the altered response in the diabetic myocardium can be reversed by activation of protein kinases PKC and p38MAPK, which signal downstream of the mitoK$_{ATP}$ channels [6]. Thus, activation of these kinases can be of therapeutic clinical use by overcoming the potential

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unresponsiveness of the diabetic myocardium to protective interventions. However, both kinases, PKC and p38MAPK, possess different isoforms whose functions are not fully elucidated. Furthermore, the use of nonspecific pharmacologic activators of these kinases may result in unwanted side effects, all of which represent considerable limitations for the clinical use of these as therapeutic targets.

Recently, we have observed that neuronal nitric oxide synthase (nNOS) is an important messenger in the signal transduction pathways of both the ischemic injury and protection in the mammalian myocardium, so that its inhibition was cardioprotective and its presence/activation was required for IP to induce protection [10]. Interestingly, the protective effect of suppressing NOS activity was replicated by the exogenous administration of nitric oxide (NO), an action that was located downstream of the mitoK$_{ATP}$ channels but upstream of p38 MAPK [10]. Theoretically, the location of exogenous NO and nNOS action beyond the mitoK$_{ATP}$ channels made them potentially useful to overcome the unresponsiveness of the diabetic myocardium to protective interventions. Indeed, the manipulation of the NO metabolism can be more physiologic and specific than the use of unspecific protein kinase activators so that a host of potential side effects could be avoided. Therefore, the aim of the present study was to investigate whether the unresponsiveness of diabetic myocardium to protection can be overcome by modulation of NO metabolism.

MATERIALS AND METHODS

Study Patients

The right atrial appendage from type II diabetic patients undergoing elective heart surgery for coronary bypass surgery or aortic valve surgery was obtained prior to the initiation of cardiopulmonary bypass. Previously, we have observed in our laboratory that the responses of the atrial myocardium from patients with type I and type II diabetes mellitus to ischemia/reoxygenation and IP are identical (unpublished data). Blood glucose levels were well controlled in all patients prior to and during surgery. Patients with atrial fibrillation, cancer, poor LV function (EF $<$ 30%), or with additional surgical procedures or those being treated with opioid, catecholamines, or the K$_{ATP}$ channel opener nicorandil were excluded from the study. The study was conducted according to Declaration of Helsinki principles and approval was obtained from the local ethics committee. All participants provided written consent.

Experimental Preparation

The experimental preparation has been previously described and fully characterized [11]. Briefly, upon harvesting, samples were immediately immersed in cold (4°C) Krebs/Henseleit/Hepes (KHH) buffered medium containing (in mM) NaCl 118, KCl 4.8, $NaHCO_3$ 27.2, KH_2PO_4 1, $MgCl_2$ 1, $CaCl_2$ 1.25, glucose 10, and Hepes 20 pre-bubbled with 95% O_2/5% CO_2 at pH of 7.4. Tissues were immediately sectioned manually with a skin-graft blade (Swann-Morton Ltd., Sheffield, UK) to slices of 30–40 mg weight and 300–500 μm thickness. The tissue and the apparatus were kept wet during sectioning with cold and oxygenated medium. After this, muscles were equilibrated under normothermic aerobic conditions (95% O_2/5% CO_2) for 50–60 min, and then subjected to 90 min of simulated ischemia at 37°C, obtained by continuously bubbling the media with 95% N_2/5% CO_2 in the absence of glucose and at pH 6.8 followed by 120 min reoxygenation. Time matched aerobic controls were used in every experiment.

For the induction of IP, muscles were subjected to 5 min ischemia followed by 5 min reoxygenation prior to induction of the 90 min of ischemia. Reagents were incubated with the muscles prior to ischemia, during ischemia, or during reoxygenation in the manner and at the doses described below in the study protocol.

Assessment of Tissue Injury

Tissue injury was assessed by measurement of creatine kinase (CK) release into the media during the 120 min reoxygenation period. The enzyme activity was measured by an ultraviolet method based on the formation of NADP employing a commercial assay kit (30-3060/R2; Abbott Laboratories, Diagnostic Division, Kent, UK) and a 96-well flat-bottom micro plate (Costar, Corning Life Sciences, Lowell, MA). In this assay, NADP is reduced to NADPH and absorbance at 340 nm is measured using a spectrophotometer (Benchmark; Bio-Rad Laboratories, Hercules, CA). Results were expressed as IU/mg wet weight.

Assessment of Cell Death

At the end of the experimental protocol, tissues were incubated for 15 min at room temperature on the rocker with 20 μg/mL propidium iodide (PI) (Sigma-Aldrich, Dorset, UK) in phosphate buffered saline (PBS) at pH 7.4 to identify the necrotic nuclei. Muscles were then washed with PBS twice for 5 min each time before fixation in 4% paraformaldehyde. They were kept over night at 4–10°C and then transferred to sucrose 30% until the tissue sank. All the above steps were performed in the dark to avoid exposure to light. Following this, the muscles were embedded with optical cutting temperature embedding matrix (Tissue-Tek; Agar Scientific Ltd., Essex, UK). Frozen sections were then cut at 7μm thickness in a Bright cryotome (model OTF) at $\sim$ –25°C, and sections were collected on SuperFrost Plus slides (Menzel Glasser, Braunschweig, Germany). The slides were then kept at –80°C.

To assess apoptosis, the slides were brought from –80°C to room temperature, washed with 20 mM PBS, and then permeabilized for 1 min in a microwave oven at 850 watts in 200 mL of 0.1% Triton X-100 in 0.1M Tri-sodium citrate buffer at pH 6.0. After this, the slides were rapidly cooled by adding 80 mL distilled water and transferred to 20 mM PBS solution. In addition, they were immersed into 3% bovine serum albumin (Sigma Aldrich, Dorset, UK) in 0.1 M Tris-HCl buffer with 20% fetal bovine serum (Hyclone, Logan, UT) at pH 7.5 for 30 min to block unspecific labeled activity. The terminal deoxynucleotidyl transferase (TdT) was used to incorporate fluorescein (FITC) labeled dUTP oligonucleotides to DNA strand breaks at the 3'-OH termini in a template dependent manner (TUNEL technique) for 90 min at 37°C in a humidity chamber using a commercially available kit (Roche Diagnostics, Penzberg, Germany).

To distinguish the total number of nuclei, sections were counterstained with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR) in PBS for 1 min. Then the slides were washed three times for 5 min each in PBS. To reduce photobleaching, the sections were mounted with anti-fade solution (Prolong Antifade kit; Molecular Probes) and covered with coverslips (Menzel Glasser, Braunschweig, Germany).

A fluorescent microscope (Axiovert 200M, Zeiss fluorescent microscope; Göttingen, Germany) at 40× magnification was use to assess necrosis and apoptosis. At least 10 fields per section and one section per staining were examined for each experiment. The fields were measured following the horizontal and vertical axes of the sections. PI and