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Duration of the Reoxygenation Interval Applied before Ischemic Post-conditioning: Fine-Tuning the Protocol for Human Myocardium

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Abstract

Ischemic post-conditioning (IPostC) is a cardioprotection strategy applied after prolonged ischemia. In an *in vitro* model of human myocardium we previously demonstrated that one cycle of 120 s of ischemia is the most protective; however the optimal duration of the reperfusion interval between prolonged ischemia and the application of IPostC have not been determined. To investigate the importance of this reperfusion interval, myocardial muscles from the right atrial appendage of 26 patients were subjected to 90 min of ischemia followed by 120 min of reoxygenation. IPostC was induced by four different reperfusion intervals (30, 60, 120 and 180 s) followed by 120 s of ischemia. Lactate dehydrogenase leakage and caspase 3 activity were measured to assess cell injury and apoptosis, respectively. The results showed that intervals of 120 and 180 s were more protective than those of 30 and 60 s, although protection was obtained in only approximately one-third of the patients. Importantly, the muscles from patients receiving nitric oxide donors as anti-anginal agents were not protected by any of the IPostC protocols used. In conclusion, the present study demonstrates the importance of the duration of the reoxygenation interval before the application of IPostC, with 120 and 180 s conferring the greatest protection. This finding is relevant for the design of future studies on the clinical utility of IPostC and on the investigation of the underlying protective mechanisms.

Keywords: Ischemic post-conditioning; Human myocardium; Ischemic injury; Apoptosis

Introduction

Cardiovascular diseases remain the main cause of death and disability in the world [1]. In patients suffering myocardial infarction, the infarct size is a major determinant of ventricular remodeling and the most important determinant of heart failure [2]. Therefore, therapeutic efforts are aimed at limiting the infarct size, usually by early reperfusion through percutaneous coronary intervention or intravenous thrombolysis. Both therapies are effective in preventing post-infarction heart failure and improving survival [3,4]. However, reperfusion after prolonged ischemia also produces a paradoxical myocardial injury that may limit the efficacy of reperfusion therapies [5]. The detrimental effect of reperfusion injury can be counteracted by interventions such as ischemic post-conditioning (IPostC), which consists of brief repetitive coronary occlusions during the early reperfusion period. IPostC was first described by Zhao et al. in a dog model of myocardial infarction [6]. In this study, three cycles of 30 s of reperfusion/ischemia each after 60 min of ischemia followed by reperfusion reduced infarct size to a degree similar to ischemic preconditioning (IPreC), a phenomenon that renders the myocardium more resistant to an ischemic insult by the previous application of short periods of ischemia [7]. However, the results from other animal models and clinical studies on the efficacy of IPostC have been controversial, as benefits [8-10], no effect [11-13] and detrimental effects [14,15] have all been described. One reason for these variable results may be the use of different IPostC protocols. Using an *in vitro* model of ischemia/reoxygenation of human myocardium, our laboratory reported that the most effective IPostC protocol was one 120 s cycle of reperfusion/ischemia after 90 min of normothermic global ischemia [16]. However, the optimal time of the interval between the termination of prolonged ischemia and the application of the short ischemia of the IPostC protocol, a time when reperfusion injury is most likely, remains unclear. Hence, the aim of the present study was to investigate the most effective duration of the reoxygenation period within the IPostC protocol in the human myocardium.

Methods

The study was approved by the local ethics committee and each patient signed the consent to participate (ID-RTF065) in the study. Right atrial appendages were collected from 26 patients undergoing elective cardiac surgery, without any exclusion criteria other than emergency surgery. Demographic data, the presence of risk factors, and the treatment history of the patients were recorded.

Experimental preparation and study protocol

Right atrial appendages were obtained immediately before the patients underwent cardiopulmonary bypass. The tissues were transferred to the laboratory in Krebs Henseleit HEPES (pH 7.4) buffer [118 mM NaCl, 4.8 mM KCl, 27.2 mM NaHCO$_3$, 1.2 mM MgCl$_2$, 1.0 mM KH$_2$PO$_4$, 1.25 mM CaCl$_2$, 10 mM glucose, 10 mM HEPES (Sigma-Aldrich, USA)] at 4°C. Connective and adipose tissues were discarded and the muscles were sliced to obtain 300-500 μm stions using a surgical skin-graft knife (Swann-Morton, Sheffield, England). The muscle slices were then equilibrated for 40 min in Henseleit

HEPES buffer (pH 7.4), oxygenated by bubbling the medium with 95% O_2/5% CO_2, and maintained at 37°C throughout the experiments. Simulated ischemia was induced by bubbling the medium with 95% N_2/5% CO_2 (pH 6.8) and replacing d-glucose by 2-deoxy-d-glucose, as described previously [17].

The experimental protocol is depicted in Figure 1. Briefly, samples were subjected to 90 min of normothermic ischemia followed by 120 min of reperfusion. One subset of muscles was subjected to ischemia/reperfusion (I/R) alone. The other muscles were subjected to a single cycle of IPostC in which the duration of ischemia was fixed at 120 s, a time previously shown to be the most effective IPostC protocol in our laboratory [16], and preceded by different reoxygenation intervals: 30, 60, 120, or 180 s. Samples not subjected to ischemia and perfused aerobically during the entire experimental period served as aerobic controls (AC).
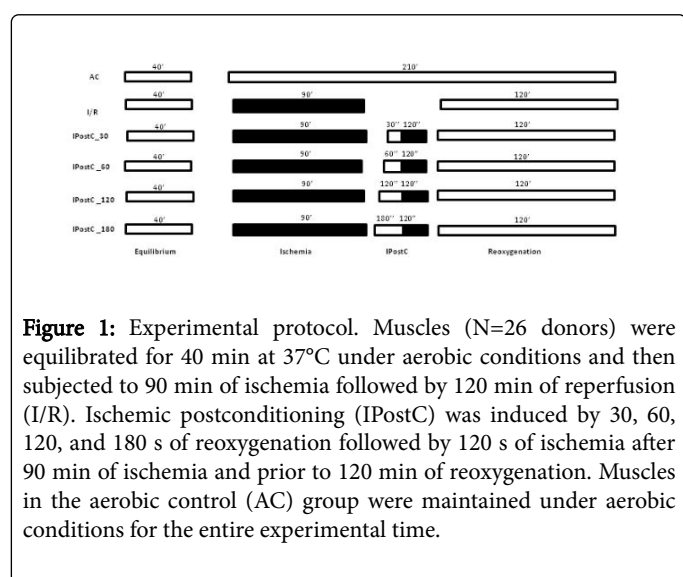

Figure 1: Experimental protocol. Muscles (N=26 donors) were equilibrated for 40 min at 37°C under aerobic conditions and then subjected to 90 min of ischemia followed by 120 min of reperfusion (I/R). Ischemic postconditioning (IPostC) was induced by 30, 60, 120, and 180 s of reoxygenation followed by 120 s of ischemia after 90 min of ischemia and prior to 120 min of reoxygenation. Muscles in the aerobic control (AC) group were maintained under aerobic conditions for the entire experimental time.

Assessment of tissue injury

Tissue injury was assessed by measuring the leakage of lactate dehydrogenase (LDH) into the incubation medium during the 120 min reoxygenation period using a kinetic method based on the reduction of NADP to NADPH. The absorbance of the samples was read at 340 nm using a Multiskan FC plate reader (Thermo Fisher Scientific, Waltham, USA) and the results were expressed as arbitrary units (AU)/g wet tissue.

Assessment of apoptosis

Tissue apoptosis was determined at the end of the experiment by measuring caspase 3 (C3) activity using a colorimetric method according to the suppliers description (BioVision, Milpitas, USA). The tissues were homogenized (Omni International, Kennesaw, USA) and the absorbance was measured at 405 nm using a Multiskan FC plate reader (Thermo Fisher Scientific). The results were expressed as ng active C3/mg protein.

Statistical analyses

Continuous variables were expressed as the means ± standard error of the mean and compared using the Wilcoxon test. Pearson's correlation was used to evaluate the association between myocardial protection and ischemic injury. Contingency tables were used to study the effect of donor-related factors, including concomitant cardiac pathologies, associated co-morbidities, and medical treatment. All statistical analyses were performed using SPSS 20 and GraphPad Prisma 6. A p value<0.05 was considered to be statistically significant.

Results

Table 1 shows the demographic data, the type of heart disease and the associated comorbid conditions of donor patients.

N=26	
Sex	
Female	9 (34.6%)
Male	17 (65.4%)
Age	
>60	14 (53.8%)
≤ 60	12 (46.2%)
CAD	12 (46.2%)
AVD	16 (61.5%)
MVD	10 (38.5%)
TVD	7 (26.9%)
Ostium sundum ASD	1 (3.8%)
LVEF	
>40%	26 (100%)
≤ 40%	0 (0%)
AF	4 (15.4%)
Obesity	7 (26.9%)
Dyslipidemia	11 (42.3%)
Hypertension	19 (73.1%)
Diabetes	7 (26.9%)
Smoker	5 (19.2%)

Table 1: Demographic data, type of heart disease and associated: comorbid conditions of donor patients; AF: Atrial Fibrillation; ASD: Atrial Septal Defect; AVD: Arterial Vascular Disease; CAD: Coronary Artery Disease; LVEF: Left Ventricle Ejection Fraction; MVD: Mitral Valve Disease; TVD: Tricuspid Valve Disease.

Figure 2A shows that the LDH leakage mean values of muscles subjected to reoxygenation intervals of 120 and 180 s before the application of IPostC were not significantly different from the values seen with muscle subjected to I/R alone; however, LDH values were significantly more elevated in the groups subjected to reoxygenation intervals of 30 and 60 s, this indicating an increase in tissue damage. Figure 2B shows that similar results were obtained for C3 activity with the shortest periods of reoxygenation affording the highest levels of apoptosis.
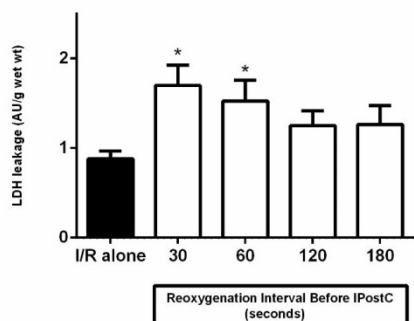
Figure 2A: Effect of the duration of the reoxygenation interval before ischemic postconditioning (IPostC) on lactate dehydrogenase (LDH) leakage of human right atrial myocardium (N=26 donors) subjected to 90 min of ischemia followed by 120 min of reoxygenation. IPostC was induced using 30, 60, 120, and 180 s intervals of reoxygenation prior to 120 s of ischemia. *$p < 0.05$ vs. the ischemia/reoxygenation (I/R) alone group.
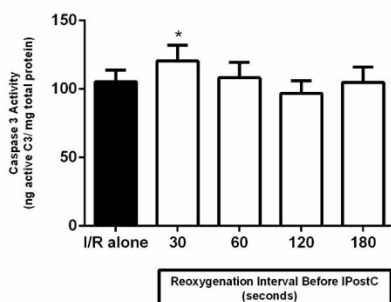

Figure 2B: Effect of the duration of the reoxygenation interval before ischemic postconditioning (IPostC) on caspase 3 activity of human right atrial myocardium (N=26 donors) subjected to 90 min of ischemia followed by 120 min of reoxygenation. IPostC was induced using 30, 60, 120, and 180 s intervals of reoxygenation prior to 120 s of ischemia. *$p < 0.05$ vs. the ischemia/reoxygenation (I/R) alone group.

Figures 3A-3D shows the individual results for LDH leakage in all the study groups. They demonstrate that IPostC with a reoxygenation interval of 60, 120 and 180 s reduced LDH leakage below the mean I/R alone group value (eg: protective) in approximately one-third of the muscles. However, LDH leakage was reduced in only 10% of the muscles treated with 30 s reoxygenation interval.
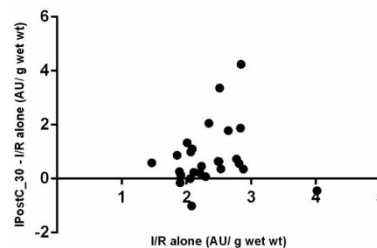

Figure 3A: Plot of all individual (N=26 donors) lactate dehydrogenase (LDH) leakage values in the ischemic postconditioning (IPostC) groups with reoxygenation interval of 30 s before the 120 s period of ischemia. The IPostC-I/R alone values (protection) were plotted against the corresponding I/R alone values.

Figure 3B: Plot of all individual (N=26 donors) lactate dehydrogenase (LDH) leakage values in the ischemic postconditioning (IPostC) groups with reoxygenation interval of 60 s before the 120 s period of ischemia. The IPostC-I/R alone values (protection) were plotted against the corresponding I/R alone values.
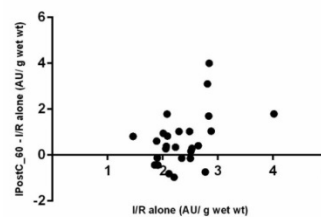

Figure 3C: Plot of all individual (N=26 donors) lactate dehydrogenase (LDH) leakage values in the ischemic postconditioning (IPostC) groups with reoxygenation interval of 120 s before the 120 s period of ischemia. The IPostC-I/R alone values (protection) were plotted against the corresponding I/R alone values.
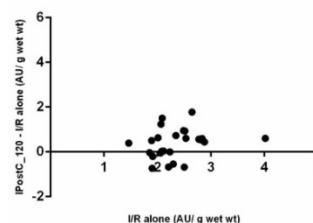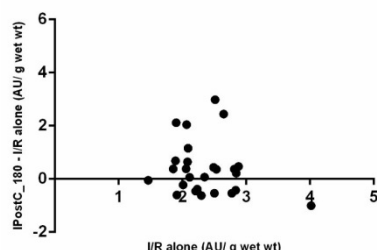
Figure 3D: Plot of all individual (N=26 donors) lactate dehydrogenase (LDH) leakage values in the ischemic postconditioning (IPostC) groups with reoxygenation interval 180 s before the 120 s period of ischemia. The IPostC-I/R alone values (protection) were plotted against the corresponding I/R alone values.

An analysis of the influence of concomitant cardiac pathologies, associated comorbid conditions, and the medical treatments received by the muscle donors on the response to IPostC revealed a significant negative relationship between IPostC and patients receiving nitric oxide (NO) donors (data not shown). In none of these patients the myocardium was protected by the most effective IPostC protocol.

Discussion

Since the first report, in 2003, that IPostC induced by three 30 s cycles of reperfusion and ischemia can reduce infarct size by 44% in anesthetized open-chest dogs with a left anterior descending artery occluded for 60 min [6], the beneficial effect of this type of intervention has been demonstrated in several animal models [18] and in man [19-21]. However, other investigators have reported no benefit [11-13] or even increasing of the ischemic damage [14,15]. It is worth noting that in the induction of IPostC the number of cycles and their duration varied and none of the studies examined the importance of the duration of the reoxygenation interval between the ischemic insult and IPostC application. Reperfusion injury, which includes calcium overload and the production of oxygen free radicals, occurs during the first few minutes of reperfusion [6,22-24], and therefore it is of critical importance to determinate what is the optimal reoxygnenation interval for the application of IPostC. Our study is the first to demonstrate that the human myocardium is better protected by an IPostC protocol with a 120 or 180 s reoxygenation interval than when an interval of 30 or 60 s is used. It also confirms our previous finding [16] that despite this only approximately one-third of the human myocardium samples obtained from patients undergoing cardiac surgery can be protected by the best IPostC protocol.

The present study also showed that the mechanisms underlying the beneficial effect of IPostC include a decrease in tissue injury, as assessed by LDH leakage, and a reduction of apoptosis, as determined by C3 activity. These findings in human myocardium are supported by a study performed in primary cultured neonatal rat cardiomyocytes exposed to 3 h of hypoxia followed by 6 h of reoxygenation and then to three 5 min cycles of reoxygenation/hypoxia [25]. The decrease in cardiomyocyte apoptosis was shown to be mediated by reductions in the release of tumor necrosis factor-α and in C3 expression, via inhibition of the JNKs/p-38 signaling pathway. In a study of patients with acute myocardial infarction undergoing percutaneous coronary intervention, IPostC also resulted in a reduction of apoptosis, as measured by the serum concentration of sFas and sFas [26].

Importantly, none of the myocardial slices from patients treated with NO donors was protected by IPostC. Fakete et al. [27] also reported the loss of protection by IPostC in isolated rat hearts in which nitrate tolerance was induced by the administration of nitroglycerin 3 days before the ischemic insult. Similar findings were obtained in a rabbit model; in that study, nitrate tolerance abolished the protection afforded by IPreC [28,29]. Together, these results highlight the important role of NO in the intracellular signal transduction mechanism of IPreC and IPostC [30,31].

In conclusion, our study showed that the duration of the reoxygenation interval before the application of IPostC is important in optimizing the protection of human myocardium against I/R-induced injury and that, in the *in vitro* model used, intervals of 120 and 180 s of reoxygenation confer the best protection. These findings do not support a wide clinical application of IPostC until the most effective protocol is fully defined in clinical settings and the patients that can be benefit from the treatment are identified. This approach is justified by the results recently published of the DANAMI-3-iPOST clinical trial [13] in which routine IPostC during primary angioplasty failed to reduce a composite outcome of death and hospitalization for heart failure in 1234 patients with an acute myocardial infarction. It is clear that further laboratory research will be required for a better understand of the mechanism of protection by IPostC that would help to refine the much needed interventions of effective therapies at the time of reperfusion.

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