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Remote Ischemic Preconditioning Decreases Adhesion and Selectively Modifies Functional Responses of Human Neutrophils

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Objectives. **Preconditioning of cells or organs by transient sublethal ischemia-reperfusion (IR), termed ischemic preconditioning (IPC), protects the cell or organ from a subsequent prolonged ischemic insult. The mechanisms of this effect remain to be fully elucidated. We have recently reported that IPC of a forearm results in alterations in gene expression profiles of circulating polymorphonuclear leukocytes. The goal of the current study was to determine if the observed changes in gene expression lead to functional changes in neutrophils.**

Methods. **We examined the effect of repetitive transient human forearm ischemia (three cycles of 5 min ischemia, followed by 5 min of reperfusion) on the function of circulating neutrophils. Neutrophil functions were examined before, after 1 d, and after 10 d of daily transient forearm ischemia. To modulate IR-induced inflammation the neutrophils were stimulated with N-formyl-methionyl-leucyl phenylalanine (FMLP) and lipopolysaccharide (LPS).**

Results. **Neutrophil adhesion was significantly decreased on day 1 and remained low on day 10 ($P = 0.0149$) without significant change in CD11b expression. Phagocytosis was significantly suppressed on day 10 compared with day 0 ($P < 0.0001$). Extracellular cytokine levels were low in the absence of an exogenous stimulus but stimulation with LPS induced significant changes on day 10. We observed a trend in reduction of apoptosis on day 1 and day 10 that did not reach statistical significance ($P < 0.08$).**

Conclusion. **This study indicates that repetitive IPC of the forearm results in substantial alterations in neutrophil function, including reduced adhesion, exocytosis, phagocytosis, and modified cytokine secretion.**

Key Words: **remote ischemic preconditioning; neutrophil function; inflammatory response; adhesion; phagocytosis.**

INTRODUCTION

Ischemic preconditioning (IPC), induced by transient sublethal ischemia, protects the cell or organ from a subsequent prolonged ischemic insult [1]. More recently, transient tissue ischemia in a remote area has been shown to confer protection of another organ subsequently subjected to potentially lethal ischemia [2]. This intriguing mode of protection against ischemia-reperfusion (IR) injury is termed remote ischemic preconditioning (rIPC). We have previously shown that transient limb ischemia protects against a systemic inflammatory response consequent to cardiopulmonary bypass [3], substantially reduces the extent of myocardial infarction after coronary occlusion [4], modifies coronary blood flow and resistance [5], and reduces myocardial IR injury after heart transplantation [6] in porcine models. More recently, we have reported the first application of rIPC in patients undergoing open-heart surgery [7], with evidence of reduced cardiac damage and systemic inflammatory response in treated patients. Although the exact mechanism of the protection afforded by rIPC remains incomplete, we have also demonstrated, in humans, that the rIPC stimulus decreases expression of a portfolio of proinflammatory genes in circulating leukocytes [8].

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Neutrophils play a key role in the IR injury [9, 10]. Neutrophil-mediated tissue damage is dependent on the number of neutrophils infiltrating the postischemic tissue via the process known as transendothelial migration. Neutrophil transendothelial migration, in turn, is influenced by the ability of circulating neutrophils to adhere to the damaged endothelium. Neutrophil adhesion, a crucial element of IR injury, is a two-stage process of selectin-mediated loose adhesion and integrin-mediated firm adhesion. One of the key integrins in the firm adhesion is the CD11b receptor. Expression of the CD11b receptor is directly related to the extent of the postoperative IR injury [11,12]. Interestingly, in a previous experimental study, rIPC attenuated both endothelial dysfunction (abnormal flow mediated dilation) and prevented the increase in neutrophil CD11b expression in humans subjected to 40 min of forearm ischemia followed by reperfusion [13].

The aim of the current study was to determine if our previous observation of the effect of rIPC on neutrophil gene expression correlated with functional changes in the leukocytes. We therefore examined the effect of repetitive transient human forearm ischemia on selected aspects of neutrophil function.

MATERIAL AND METHODS

Subjects and Blood Samples

We performed a longitudinal study using the rIPC protocol as previously described [13] in five healthy adult male volunteers who were taking no medications (mean age, 37.5 y). Briefly, one forearm was made ischemic by inflating a blood pressure cuff to 200 mm Hg for three 5-min periods, separated by 5 min of reperfusion. This protocol was repeated daily for 10 d. Venous blood was drawn from the contralateral arm prior to the ischemic stimulus (day 0) and on days 1 and 10 after the stimulus. All samples were collected before the rIPC procedure on the day of sampling. Samples were collected in standard sterile tubes with EDTA anticoagulant (Vacutainer; Preanalytical Solutions, Franklin Lakes, NJ) and transported on ice for immediate assessment of leukocyte function. Neutrophils were isolated from whole blood using dextran sedimentation and discontinuous plasma-Percoll gradients (Amersham Biosciences, Uppsala, Sweden) as described previously [14]. The separation procedure was complete within 2 h and the cells were used immediately after isolation for the experiments described. Neutrophil purity was >98% and viability was >97% using Trypan blue exclusion. The functional integrity and nonactivated state of neutrophils isolated has been validated in previous publications [15].

The experimental design was approved by the Hospital for Sick Children Research Ethics Board (file number 1000000626, November 11, 2002).

Assessment of Leukocyte Function

We assessed a range of functional responses in peripheral blood neutrophils during the study including adhesion to serum-coated plastic, cell surface expression of CD11b, exocytosis of primary and secondary granules as assessed by surface expression of granule markers and extracellular release of granule enzymes, cytokine production, apoptosis, activation of the respiratory burst oxidase, and phagocytic ability.

Adhesion

Adhesion of neutrophils was measured as the percentage (%) of cells that adhered to tissue culture wells coated with fetal bovine serum. In addition, surface expression of CD11b (Mac-1), a pivotal adhesion molecule, was assessed by measuring fluorescence intensity of neutrophils labeled with FITC-conjugated anti-CD11b monoclonal antibodies (Serotec, Oxford, UK) as described previously [13].

Secretion

Secretion of primary and secondary granule contents (exocytosis) was assessed by flow cytometry measuring surface expression of CD63 and CD66b, respectively, using FITC-conjugated antibodies (Serotec). Secretion of the cytokines TNFα, IL-1β, IL-6, and IL-10 were measured using a multiplex fluorescent bead assay (LINCOplex; LINCO, New York, NY) using a Luminex in response to stimulation with LPS (100 ng/mL) (Sigma, Oakville, ON, Canada) or vehicle control for 6 h and 24 h at 37°C.

Apoptosis

Apoptosis was assessed using a combination of propidium iodide and annexin V-FITC (R and D Systems, Minneapolis, MN) fluorescence staining with quantification by flow cytometry as previously described [16].

Oxidant Production

Oxidant production (NADPH oxidase) by neutrophils was assessed by flow cytometry using the fluorescent dye dihydrorhodamine (DHR) as previously described [17]. Peripheral blood neutrophils were pretreated with cytochalasin B (5 μM) for 10 min followed by exposure to N-formyl-methionyl-leucyl phenylalanine (FMLP 10^{-7}M) for an additional 10 min. Alternatively, cells were exposed to 10^{-6} M phorbol myristate acetate (PMA) for 20 min as a positive control. The cells were then incubated with 10^{-5} M dihydrorhodamine (Invitrogen, Carlsbad, CA) for an additional 5 min at 37°C followed by fixation with 1.6% paraformaldehyde (Canemco Inc., St. Laurent, Quebec). The fluorescence of the cell associated reduction product, rhodamine 1-2-3, was evaluated by flow cytometry as a measure of oxidant production.

Phagocytosis Assay

IgG-coated prey was constructed as described [18]. Briefly, 100 μL of sheep erythrocytes 10% solution (Cappel, West Chester, PA) was washed twice in PBS, incubated with 2 μL of rabbit anti sheep erythrocyte IgG (INC55806; Cappel) for 1 h, and washed twice in PBS. Neutrophils were washed twice with Hanks buffered salt solution followed by addition of the phagocytic prey at a ratio of 20/1 and allowed to interact and bind to the neutrophils for 5 min at 37°C. The cells were washed to remove unbound prey and incubated at 37°C for an additional 15 min to allow phagocytosis to proceed. The assays were terminated by cooling the cells by washing with ice cold PBS without calcium and magnesium. Following incubation, hypotonic lysis of the extracellular erythrocytes was achieved by addition of water for 30 s, followed by immediate replacement with calcium and magnesium-free PBS. The coverslips were mounted on Attofluor cell chambers (Invitrogen Canada, Inc.) and quantification of phagocytosis conducted using an inverted microscope (Leica DM-IRB, Wetzler, Germany).

Data Analysis

Data were analyzed using a paired *t*-test for comparison between two conditions on a same sample, and repeated measure of analysis of variance (ANOVA) with *post hoc* analysis by Student-Newman-Keuls multiple comparison test, or two way ANOVA using GraphPad