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Research paper

Gene expression profile of vascular ischemia-reperfusion injury in rhesus monkeys

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ABSTRACT

The vascular system particularly endothelium is sensitive to ischemia-reperfusion (I/R) injury, which is a big challenge in surgical practices and many vascular disorders. In the present study, we reported the global gene expression changes in a 2-h ischemia and 4-h reperfusion injury induced in the hind limb vessels of rhesus monkeys (*Macaca mulatta*) using microarray technique. Results: The histological results showed abnormal morphology of endothelial cells after 2-h ischemia and the hematological detection found slightly extension of coagulation time after I/R treatment. Furthermore, we found distinct alterations in gene expression patterns during I/R process. These identified genes are mostly involved in inflammation, immune response, apoptosis, and cell stress signaling pathways. The significantly up-regulated genes included *IL-6*, *regulator of G-protein signaling 8*, *selectin E*, *and metallothionein 2A*, et al. Whist, the robustly down-regulated genes included *NECAP endocytosis associated 2*, *transglutaminase 2*, *and fibronectin 1*, et al. Conclusion: Our results indicate that inflammation, primarily characterized by gene expression changes of cytokines and chemokines is the most important event in the early stage of I/R injury in blood vessels.

1. Introduction

The reperfusion of bloodstream to an organ in ischemia is essential for its viability and functional recovery, however the detrimental side of this scenario is that the arrival of blood oxygen will cause additional lesions, which is known as ischemia-reperfusion (I/R) injury (Gourdin et al., 2009). All oxygen dependent cells that rely on an uninterrupted blood supply are vulnerable to I/R injury. Vascular system, particularly the endothelium is sensitive to I/R injury. The arterial clamping/unclamping in vascular surgery, cardiac surgery with extracorporeal circulation, and organ transplantation are some typical I/R situations in clinical operating practices. Disorders including myocardial infarction, stroke, and peripheral vascular disease are also essentially characterized by I/R (Kalogeris et al., 2012). I/R on endothelial cells results in a range of pathophysiological deteriorations. For instance, ischemia followed by reperfusion remarkably decreases endothelium-dependent relaxations of coronary arteries (Laude et al., 2004). I/R injury in solid organ transplantation, the unavoidable event in organ storage and surgery process, is associated with chronic graft dysfunction (CGD) and reduced long-term survival of the transplanted organs (Barbari et al., 2001; Menke et al., 2014; Zhai et al., 2013). Intriguingly, endothelial inflammation and dysfunction is proven to play an important role in this scenario (Basile and Yoder, 2014; Bruneau et al., 2015; Coito, 2011; Rose, 2004). Additionally, acute limb vascular ischemia and reperfusion means serious challenge in clinical treatment of trauma. Ischemia-reperfusion causes complex alterations in hemodynamics, microcirculation, as well as endothelial function, and accounting to local and systemic metabolic changes after limb I/R (Szokoly et al., 2009).

Abbreviation: I/R, ischemia-reperfusion; CGD, chronic graft dysfunction; ROS, reactive oxygen species; APTT, activated partial thromboplastin times; HE, hematoxylin-eosin; qPCR, quantitative real-time PCR; RMA, Robust Multichip Analysis; CXCL1/3, chemokine (C-X-C motif) ligand 1/3; SERPINA3, serpin peptidase inhibitor, clade A, member 3; PTX3, pentraxin 3; CCL4L1, chemokine (C-C motif) ligand 4-like 1; SELE, selectin E; TGM2, transglutaminase 2; IL-6, interleukin-6; IL-8, interleukin-8; IL-1β, interleukin-1β; NECAP, NECAP endocytosis associated 2; CK, creatine kinase; WBC, white blood cells; PT, prothrombin times; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; MT, metallothionein; MT-1F, metallothionein-1F; MT-II, metallothionein-II; CCVs, clathrin-coated vesicles; HUVECs, human umbilical vein endothelial cells; vWF, von Willebrand factor; RNA, ribonucleic acid; cDNA, complementary DNA.

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Although I/R has been studied for decades, the underlying mechanisms has not been fully elucidated. Current knowledge of I/R links to multiple factors including oxidative stress, mitochondrial dysfunction, inflammatory responses, and apoptosis. During prolonged ischemia, decreases of ATP levels and intracellular pH lead to calcium over influx in plasma and mitochondria, resulting in cell swelling and rupture, and cell death by apoptosis, autophagy and necrosis. Reperfusion of blood recovers the oxygen levels, but the generation of excess reactive oxygen species (ROS) and proinflammatory neutrophils infiltration exacerbate ischemia injury (Kalogeris et al., 2012). Exploring the molecular and cellular mechanisms involved in vascular and endothelial I/R injury would benefit our better understanding of I/R related diseases and provide further targets for pharmaceutical and clinical intervention.

I/R has been experimentally investigated mostly in small-animal models, focusing on the pathological changes in targeted organs such as brain, heart, liver, intestine and kidney (Gonzalez et al., 2015). Dedicated attentions to the specific changes on vascular endothelium are lacking in both in vivo and in vitro studies. In addition, it is noteworthy that how poor murine models could mimic human inflammatory responses. Higher priority for translational medical research to focus on more complex human conditions rather than relying on mouse models is encouraged (Seok et al., 2013). Taken this in mind, development of vascular I/R models in non-human primates for further in-depth studies are warranted.

In the present study, we provide the first report of global alterations in gene expression induced by I/R injury in the hind limb vessel of rhesus monkeys (*Macaca mulatta*). We used high-density DNA microarray technique in our study, which is capable to uncover previously undescribed genes participating in I/R injury and lead to comprehensive understanding of the multifactorial pathological process (Yoshida et al., 2002b).

2. Materials and methods

2.1. Animals

Six rhesus monkeys (*Macaca mulatta*, 3 males and 3 females, 3–4 years, and weight 4–5 kg), were obtained from Ping'an Animal Breeding Center in Chengdu, China. Monkeys were housed in clean primate facilities, and had access to food and water ad libitum, as well as seasonal fruit and vegetables. All procedures in this study were in compliance with the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals. The experimental treatment of animals were reviewed and approved by the Animal Care & Welfare Committee of West China Hospital, Sichuan University.

2.2. I/R model

In this study, the six monkeys were randomly divided into two groups. The 3 left hind limbs of group 1 were set as control group (C, $n = 3$); another 3 right hind limbs of group 1 were set as ischemia 2-h group (I_2, $n = 3$); the 3 left hind limbs of group 2 were set as ischemia 2-h followed by reperfusion 2-h group (I_2R_2, $n = 3$); and the 3 right hind limbs of group 2 were set as ischemia 2-h followed by reperfusion 4-h group (I_2R_4, $n = 3$).

Before surgery, all the animals underwent overnight fasting and were anesthetized following our previous description (Zhang et al., 2011). Animals were premedicated with ketamine (15 mg/kg, im) and midazolam (0.4 mg/kg, im). Anesthesia was induced with propofol (2 mg/kg per minute, iv) and was maintained with propofol (0.1 mg/kg per min, iv) and fentanil (2 μg/kg, iv). N2-cholinoceptor-blocking drug was administered during the operation. The vital signs of animals were monitored regularly.

Acute limb ischemia was performed as follows: under sterile conditions, the proximal portion of the femoral artery and vein including the superficial and the deep branch, as well as the distal portion of the saphenous artery were carefully exposed. The common femoral artery was completely occluded by two vessel clamps proximally, while the femoral vein stayed open. In the reperfusion groups, the femoral artery was re-opened after 2-h ischemia by withdrawing the vessel clamps. The Doppler ultrasound examinations were performed before operation, after ischemia as well as after reperfusion to confirm the well ischemia and reperfusion in the common femoral artery. In the control group, sham operation was performed. At sacrifice, about 5 cm the femoral arteries of each hind-limb were removed. Partial tissues were kept in RNAlater (Invitrogen, US) for further RNA isolation and others were kept in paraformaldehyde for histological analysis. In the end, all the animals received euthanasia.

2.3. Determination of hematological and biochemical parameters

Peripheral blood samples were collected from the ulnar vein of upper limb before operation, after ischemia and after reperfusion to investigate whether temporary limb ischemia-reperfusion results in systemic hematological and biochemical alteration or remote organ abnormalities. Biochemistry measurements were carried out on sera samples, using Olympus AU5400 autoanalyzer (Japan). Analysis included total bilirubin, alanine aminotransferase, aspartate aminotransferase, total protein, urea nitrogen, creatinine, triglyeride, cholesterol, alkaline phosphatase, gamma glutamyl transferase, creatine kinase, lactate dehydrogenase, and glucose, et al. Full blood count tests were performed using Hematology XE-2100 (Japan). Coagulation function, including prothrombin times (PT) and activated partial thromboplastin times (APTT) were determined in plasma samples using a STA-Stargo autoanalyzer (France). All of the tests were performed in the National Center for Safety Evaluation of Traditional Chinese Medicine, Chengdu, P.R.China.

2.4. Histology analysis

The femoral arteries undergoing ischemia and reperfusion were collected in paraformaldehyde for histological analysis. Tissue sections were stained with hematoxylin-eosin (HE) to observe whether pathological damages and/or immune cells infiltration happened to the vascular structure after I/R injury.

2.5. Total RNA extraction

After I/R or sham operation, the femoral arteries of each hind-limb collected in RNAlater solution were subjected to total RNA extraction. Total RNA was isolated using TRIzol Reagent (Invitrogen life technologies, US) and Ambion mirVana miRNA Isolation Kit (Ambion, life technologies, US), verified for purity and quantitation by spectrophotometry. Equal amount of three individual RNA samples from each group were pooled together before subjected to one microarray experiment, as well as qPCR.

2.6. Microarray procedures

Affymetrix Gene Chip® Rhesus Macaque Genome Arrays (Affymetrix, US) were used for microarray analysis in this study. The microarray experiments and bioinformatics analysis were performed by CapitalBio Corporation Company, China. Detailed description of Affymetrix microarray technology and procedures is available at the company website (http://www.capitalbio.com). Briefly, 100 ng purified total RNA was reverse transcribed to first and second-strand cDNA by MessageAmp™ Premier RNA Amplification Kit (Ambion, US). Then, the cDNA samples, labeled with biotin, were bond with RNA Binding Beads for purification. After cDNA fragmentation, hybridizations to microarray slides containing approximately 47,000 unique sequence-verified rhesus monkey transcripts were carried out with Hybridization, Wash and Stain kit (Affymetrix, US). The array slides were scanned