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Arginine administration increases circulating endothelial progenitor cells and attenuates tissue injury in a mouse model of hind limb ischemia/reperfusion

Kuan-Feng Hsieh M.S. [a], Juey-Ming Shih M.D., Ph.D. [b], Yao-Ming Shih M.D., Ph.D. [b], Man-Hui Pai M.S. [c], Sung-Ling Yeh R.D., Ph.D. [a,d,*]

[a] School of Nutrition and Health Sciences, College of Nutrition, Taipei Medical University, Taipei, Taiwan
[b] Department of Surgery, Cathay General Hospital, Taipei, Taiwan
[c] Department of Anatomy and Cell Biology, College of Medicine, Taipei Medical University, Taipei, Taiwan
[d] Nutrition Research Center, Taipei Medical University Hospital, Taipei, Taiwan

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ABSTRACT

Objective: This study investigated whether the administration of L-Arginine, the precursor of nitric oxide, increases the percentages of blood endothelial progenitor cells and protects against ischemia/reperfusion induced inflammatory response in a mouse model of hind-limb IR injury.
Method: C57 BL/6 mice were randomized to one normal-control and four ischemia/reperfusion groups. The normal-control group did not undergo an ischemia/reperfusion procedure but mice in the ischemia/reperfusion groups were subjected to 150 min of unilateral hind-limb ischemia. The ischemia/reperfusion groups were subjected to either intravenous saline or L-Arginine (300 mg/kg body weight) administration before reperfusion and then sacrificed at either 24 h or 48 h after reperfusion. Blood and muscle tissues were collected for analysis.
Results: Ischemia/reperfusion injury led to a significant decrease in the percentage of blood endothelial progenitor cells and plasma nitric oxide concentration but plasma interleukin-6 levels and gene expression of inflammatory cytokines in injured muscle tissue were elevated. In contrast to the saline groups, those with L-Arginine administration were able to maintain a normal level of blood endothelial progenitor cells. In addition, after reperfusion, concentrations of nitric oxide, matrix metallopeptidase-9, and vascular endothelial growth factor in plasma were upregulated but keratinocyte-derived chemokine and monocyte chemoattractant protein-1 messenger RNA expressions in muscle were attenuated 48 h after reperfusion. Histologic findings also demonstrated a significant reduction of ischemia/reperfusion-induced muscle injury when L-Arginine was administered.
Conclusion: A single dose of L-Arginine administration before reperfusion increases the percentage of endothelial progenitor cells and reduces the inflammatory reaction locally and systemically after ischemia/reperfusion injury.

Introduction

Limb ischemia is caused by the arterial obstruction of the lower extremity, which frequently occurs in peripheral arterial embolism, atherosclerotic thrombosis, and traumatic vascular injuries [1]. Blood reperfusion is necessary to restore the metabolic function of ischemic tissue; however, the process of reperfusion often leads to an increased production of reactive oxygen species (ROS) and neutrophil infiltration as well as induces local and systemic inflammatory reactions that may result in local and remote tissue injury [2]. The mechanisms of ischemia-reperfusion (IR) injury are multifactorial, yet endothelial cell dysfunction is considered a major contributor to IR-induced injury [3].

Recent evidence suggests that endothelial progenitor cells (EPCs) are important to maintain vascular endothelial integrity and restore microcirculation during catabolic conditions [4]. EPCs are bone marrow-derived cells that are capable of differentiating

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* Corresponding author. Tel: +8862-27361661 ext. 6547; fax: +8862-66369175.
E-mail address: Sangling@tmu.edu.tw (S.-L. Yeh).

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into mature endothelial cells (EC) [5]. Several angiogenetic factors that are involved in the recruitment of EPCs include vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF-1), and matrix metallopeptidase (MMP)-9, which all seem to be activated in response to conditions of deprived tissue oxygen and nutrients [6]. Furthermore, EPCs have been shown to be involved in wound healing, tissue regeneration, and neovascularization of ischemic tissues [6,7]. A clinical study has shown that, compared with healthy controls, patients with critical limb ischemia have reduced levels of circulating EPCs [8].

Although classified as a non-essential amino acid, L-Arginine (L-Arg) has been found to possess many physiological properties with important functions. L-Arg is required for the synthesis of proteins, urea, and polyamines [9]. Also, L-Arg has been shown to exhibit immune-regulatory characteristics [10] and is considered a conditionally essential amino acid in catabolic conditions [11].

More importantly, L-Arg is the sole substrate of nitric-oxide synthase (NOS) and the precursor of nitric oxide (NO) synthesis [12]. A previous study found that the expression of VEGF messenger RNA (mRNA) in wound tissue was upregulated when animals were given L-Arg supplementation [13]. Although VEGF plays an important role in the mobilization of EPCs, the process also relies on NO bioavailability, which is L-Arg-dependent [14].

Our recent investigation also showed that L-Arg administration enhanced EPC mobilization and attenuated remote lung injury in a mice model of cecal ligation and puncture-induced sepsis [15]. In this study, we hypothesized that L-Arg administration may increase NO availability, promote EPC mobilization, and alleviate the inflammatory response that is induced by limb IR injury.

Methods

Animals

Fifty male C57 BL/6 mice (5 wk old; weight, 18–20 g) were used in the study. All mice were housed in a temperature- and humidity-controlled room with a 12-h light-dark cycle and fed a standard chow diet and tap water ad libitum for 1 wk. The care of the laboratory animals was in compliance with the 2011 Guide for the Care and Use of Laboratory Animals by National Research Council. Experiment protocols were approved by the institution's Animal Care and Use Committee at the Taipei Medical University (LAC-2015-0018).

Experimental design

Mice were randomly assigned to one normal control (NC) group and four IR groups. Mice in the NC group did not undergo the ischemic procedure and were fed a standard chow diet (Purina No. 5001). Mice in the IR groups were subjected to unilateral hind limb ischemia for 150 min followed by either 24 h or 48 h reperfusion. The induction of ischemia was performed as described by Crawford et al. [16]. Animals in the IR groups were intraperitoneally injected with Zoletil (25 mg/kg) and Rumpun (10 mg/kg). Next, a 4.5-oz orthodontic rubber band (ORB; American Orthodontics, Sheboygan, WI) was applied to the left thigh above the greater trochanter and 0.5 mL saline were subcutaneously injected for hydration upon the initial banding. The mice were kept anesthetized throughout the ischemic period.

After 150 min of ischemia, reperfusion was initiated by cutting the rubber band and the animals were allowed to recover from anesthesia. Just before the start of the reperfusion, animals were given a single dose of either L-Arg (300 mg/kg body weight) or an equal volume of saline via the tail vein. Mice in the L-Arg (A) and saline (S) groups were sacrificed at 24 h (24 A and 24 S groups) or 48 h (48 A and 48 S groups) after reperfusion with 10 mice at each time point. At the end of the respective experimental period, the mice were euthanized by cardiac puncture. Blood samples were collected in tubes that contained heparin. Gastrocnemius muscle tissues of the affected limbs were frozen in liquid nitrogen and stored at –80ºC for further analysis.

Flow cytometry for the blood endothelial-progenitor cell analysis

EPC analysis was performed in accordance with the standard settings on a multicolor BD FACS CantoII flow cytometer (BD Biosciences, San Diego, CA) and data were analyzed with BD FACSDiva version 6.1.3 software (BD Biosciences). One hundred microliter of fresh blood was incubated with fluorescein isothiocyanate-conjugated antimouse CD34 (RAM34, eBioscience, San Diego, CA), allophycocyanin-conjugated antimouse CD309 (Avas12 a1, eBioscience), and phycoerythrin-conjugated antimouse CD133 (13 A4, eBioscience). After 30 min of incubation, red blood cells were lysed with lysing buffer (PharmLyse; BD Pharmingen) and fixed in 2% paraformaldehyde before analysis. During the data analysis, the percentage of circulating EPCs was defined as $CD34^+/CD133^+/CD309^+$-positive cells, which was gated among a mononuclear cell population of 50 000 events.

Measurements in plasma

Plasma NO is an unstable substance and was converted to stable nitrite and nitrate ions using Griess reagent. Subsequently, concentrations of nitrite/nitrate were determined utilizing a commercial kit (R&D Systems, Minneapolis, MN). MMP-9, VEGF, and interleukin (IL)-6 were measured with enzyme-linked immunosorbent assay kits (eBioscience) in accordance with the manufacturer's instructions.

Measurements in muscle

Muscle samples were homogenized (1:5, w/v) in ice-cold, phosphate-buffered saline. The homogenates were centrifuged at 15 000 rpm for 20 min and the supernatants were used for the analysis of EPC-mobilizing proteins. VEGF and SDF-1 concentrations in muscle homogenates were measured with enzyme-linked immunosorbent assay kits (eBioscience) in accordance with the manufacturer's instructions.

Expression in muscle and real-time reverse-transcription polymerase chain reaction analysis

Muscle tissues were homogenized using the Trizol reagent (Invitrogen, Carlsbad, CA) to isolate total RNA. The RNA pellet was dissolved in RNase-free water and stored at –80°C for the subsequent assay. The RNA concentration was determined and quantified by measuring absorbances at 260 nm and 280 nm on a spectrophotometer. Complementary DNA (cDNA) was synthesized from total RNA using a RevertAid first-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). Reverse transcription was performed by a subsequent incubation for 5 min at 65°C, 60 min at 42°C, and 5 min at 70°C. cDNA was stored at –80°C until analyzed.

Specific mRNA genes were amplified by a real-time RT polymerase chain reaction using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with SYBR Green I as the detection format. The primers that were used in this study are listed in Table 1. The primers were purchased from Mission Biotech (Taipei, Taiwan) based on deposited cDNA sequences (GenBank database, National Center for Biotechnology Information). Amplification was carried out in a total volume of 25 μL that contained 1 x Power SYBR Green Polymerase Chain Reaction Master Mix (Applied Biosystems), 400 nM of each primer, and 100 ng

Table 1
Sequence of oligonucleotide primers used in PCR amplification

Genes	Primer sequences (5'–3')
SDF-1	F:CAGCCGTGCAACAATCTGAAG
	R:CTGCATCAGTGACGGTAAACC
VEGF	F:GATCATGCGGATCAAACCTC
	R:AATGCTTTCTCCGCTCTGAA
HIF-1α	F:CGCCTCTGGACTTGTCTCTT
	R:TTCTTCTCGTTCTCGCCGC
MCP-1	F:ACCACAGTCCATGCCATCAC
	R:TTGAGGTGGTTGTGGAAAAG
KC	F:CTTGAAGGTGTTGCCCTCAG
	R:TGGGGACACCTTTTAGCATC
GAPDH	F:GAAGGTCGGTGTGAACGGAT
	R:AATCTCCACTTTGCCACTGC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIF-1α, hypoxia inducible factor 1 alpha; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein 1; SDF-1, stromal-derived growth factor 1; VEGF, vascular endothelial growth factor.