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Dietary glutamine supplementation enhances endothelial progenitor cell mobilization in streptozotocin-induced diabetic mice subjected to limb ischemia☆

Shiau-Tsz Su[a], Chiu-Li Yeh[b], Yu-Chen Hou[c], Man-Hui Pai[d], Sung-Ling Yeh[a,e,*]

[a]School of Nutrition and Health Sciences, College of Nutrition, Taipei Medical University, Taipei, Taiwan
[b]Department of Nutrition and Health Sciences, Chinese Culture University, Taipei, Taiwan
[c]Master Program in Food Safety, College of Nutrition, Taipei Medical University, Taipei, Taiwan
[d]Department of Anatomy and Cell Biology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[e]Nutrition Research Center, Taipei Medical University Hospital, Taipei, Taiwan

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Abstract

Diabetes is a metabolic disorder with increased risk of vascular diseases. Tissue ischemia may occur with diabetic vascular complications. Bone marrow-derived endothelial progenitor cells (EPCs) constitute a reparative response to ischemic injury. This study investigated the effects of oral glutamine (GLN) supplementation on circulating EPC mobilization and expression of tissue EPC-releasing markers in diabetic mice subjected to limb ischemia. Diabetes was induced by a daily intraperitoneal injection of streptozotocin for 5 days. Diabetic mice were divided into 2 nonischemic groups and 6 ischemic groups. One of the nonischemic and 3 ischemic groups were fed the control diet, while the remaining 4 groups received diets with identical components except that part of the casein was replaced by GLN. The respective diets were fed to the mice for 3 weeks, and then the nonischemic mice were sacrificed. Unilateral hindlimb ischemia was created in the ischemic groups, and mice were sacrificed at 1, 7 or 21 days after ischemia. Their blood and ischemic muscle tissues were collected for further analyses. Results showed that plasma matrix metallopeptidase (MMP)-9 and the circulating EPC percentage increased after limb ischemia in a diabetic condition. Compared to groups without GLN, GLN supplementation up-regulated plasma stromal cell-derived factor (SDF)-1 and muscle MMP-9, SDF-1, hypoxia-inducible factor-1 and vascular endothelial growth factor gene expression. The CD31-immunoreactive intensities were also higher in the ischemic limb. These findings suggest that GLN supplementation enhanced circulating EPC mobilization that may promote endothelium repair at ischemic tissue in diabetic mice subjected to limb ischemia.

Keywords: Diabetic ischemic limb; Glutamine; Endothelial progenitor cells; Hypoxia-inducible factor-1; CD31

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia and hyperlipidemia that often associated with an increased risk of cardiovascular diseases [1]. Endothelial dysfunction accompanied by an up-regulated inflammatory reaction is the major contributing factor to the pathogenesis of diabetic vascular complications [2]. Previous study found that patients with diabetes have reduced formation of collateral vessels in the heart and peripheral tissues in response to ischemia [3]. This impaired mechanism of angiogenesis may contribute to atherosclerosis, distal limb amputation and increase mortality in chronic diabetic patients [4]. It has been shown that the regeneration of the vascular endothelium is a crucial step in the repairing process of injured blood vessels induced by diabetic glucotoxicity and lipotoxicity [5].

Endothelial progenitor cells (EPCs) are cells derived from bone marrow that are able to differentiate to mature endothelial cells (ECs) [6]. EPCs have been shown to be involved in neovascularization, wound healing, tissue regeneration and tissue remodeling [7]. Several factors are activated to recruit EPCs to ischemic sites in order to form new blood vessels. Hypoxia-inducible factor (HIF)-1 is an oxygen-dependent transcriptional activator, which plays important roles in the angiogenesis of ischemic tissues [8]. HIF-1α is necessary for the expressions of vascular endothelial growth factor (VEGF) and stromal cell-derived factor (SDF)-1 [8,9]. VEGF and SDF-1 can induce the stromal cells residing within the bone marrow to secrete matrix metallopeptidase (MMP)-9, which degrades the extracellular matrix therefore aiding the release of EPCs from bone marrow into the circulation and homing to target tissues [7]. Previous studies have shown that diabetes is associated with reduced circulating EPC numbers [10], and EPCs from diabetic patients reduced their capacity of angiogenicity *in vitro* [11]. Animal studies have revealed that the ability of EPCs to be mobilized in response to ischemia becomes

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* Corresponding author at: School of Nutrition and Health Sciences, Taipei Medical University, 250 Wu-Hsing Street, Taipei, 110, Taiwan. Tel.: +886-2-27361661x6547.
E-mail address: sangling@tmu.edu.tw (S.-L. Yeh).

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hampered in diabetes, which consequently prohibited the reparative process at sites of vascular damage [12–14].

Glutamine (GLN) is the most abundant free amino acid in our body including the plasma [15]. A previous study showed that profound GLN depletion is associated with poor clinical outcomes and suggested that replenishing the deficit with GLN supplementation may be necessary for maintaining GLN homeostasis in catabolic conditions [16]. Diabetic patients in particular exhibit significant reduction in plasma GLN level, which can lead to disturbances in the secretion and actions of insulin [17]. Also known as an essential substrate for rapidly proliferating cells, GLN regulates the proliferation of cardiac progenitor cells [18] and promotes the mobilization of circulating EPCs in septic condition [19]. At present, no study has ever investigated the effects of GLN supplementation on changes in mobilization of blood EPCs and in repair of damaged vessels in ischemic tissues in diabetes. In this study, we used a unilateral ischemic hindlimb model to investigate the impacts of GLN supplementation on EPC mobilization and ischemia-induced muscle damage in streptozotocin (STZ)-induced diabetic mice. Percentage of early and late EPCs as well as the genes and proteins related to EPC mobilization including HIF-1α, MMP-9, VEGF and SDF-1 was measured in the blood and ischemic-injured muscle tissue. The expression of antiapoptotic Bcl-xL [20], cell-protecting heat shock protein (HSP)70 [21] and peroxisome proliferator-activated receptor (PPAR)-α [22] genes in muscle tissues were also analyzed. We hypothesized that GLN intervention would enhance EPC mobilization and improve endothelium repair at the site of ischemia in diabetic animals.

2. Materials and methods

2.1. Animals

Male C57BL/6 J mice at 6–7-weeks old and weighing 18–20 g at the beginning of the experiment were used in this study. All mice were kept in a temperature- and humidity-controlled room and were fed with a standard chow diet (Purina No. 5001) [23] for 1 week before the study. Experimental diabetes was induced by a daily intraperitoneal injection of STZ [40 mg/kg body weight (BW)] for 5 consecutive days as described previously [24]. STZ was dissolved in sodium citrate buffer (0.05 M, pH 4.5) immediately before use. Ten days after the last STZ injection, blood glucose levels were measured. Mice were considered diabetic if their blood glucose levels exceeded 200 mg/dl after 5 h of fasting [25]. Verified diabetic mice were not treated with STZ or insulin during the experimental period. BW and food intake were recorded every week till the time of sacrifice. Animal care and all experiments were performed according to the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), and protocols were approved by the institutional Animal Care and Use Committee of Taipei Medical University (LAC-2015-0019).

Table 1
Composition of the experimental diets (g/kg).

Component	Control diet	Glutamine diet
Corn starch	529.5	537.84
Casein	200	149.45
L-Glutamine	0	42.21
Sucrose	100	100
Soybean oil	70	70
Fiber	50	50
Mineral mixture†	35	35
Vitamin mixture‡	10	10
L-Cysteine	3	3
Choline	2.5	2.5
Tert-butylhydroquinone	0.014	0.014

† The salt mixture contains the following (mg/g): calcium phosphate dibasic, 500; sodium chloride, 74; potassium sulfate, 52; potassium citrate monohydrate, 20; magnesium oxide, 24; manganese carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; curpric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; and chromium potassium sulfate, 0.55.

‡ The vitamin mixture contains the following (mg/g): thiamin hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride, 0.7; nicotinic acid, 3; calcium pantothenate, 1.6; D-biotin, 0.05; cyanocobalamin, 0.001; retinyl palmitate, 1.6; DL-α-tocopherol acetate, 20; cholecalciferol, 0.25; and menaquinone, 0.005.

Table 2
Sequence of oligonucleotide primers used in the PCR amplification.

Gene	Primer sequences (5′ to 3′)
GAPDH	F: GAAGGTCGGTGTGAACGGAT
	R: AATCTCCACTTTGCCACTGC
MMP-9	F: CCAGCCGACTTTTGTGGTCT
	R: TGGCCTTTAGTGTCTGGCTG
VEGF	F: GATCATGCGGATCAAACCTC
	R: AATGCTTTCTCCGCTCTGAA
SDF-1	F: CAGCCGTGCAACAATCTGAAG
	R: CTGCATCAGTGACGGTAAACC
HIF-1α	F: CGCCTCTGGACTTGTCTCTT
	R: TTCTTCTCGTTCTCGCCGC
Hsp70	F: CACCATCGAGGAGGTGGATT
	R: ACAGGGAAGATAAAGCCCACG
PPAR-α	F: CCATTGGTGATGTTGCAAAG
	R: CTTTTTCGCTGCATCAGGTT
Bcl-xL	F: CCTCCCCGACCTATGATTCAA
	R: TCAAATCTATCTCCGGCGACCA
Caspase3	F: AGTCGAAAGTGCCCACCAAT
	R: ATTCCAAAACTCGACCCGCT

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP-9, matrix metallopeptidase-9; VEGF, vascular endothelial growth factor; SDF-1, stromal cell-derived factor 1; HIF-1α, hypoxia-inducible factor 1-α; HSP70, heat shock protein 70; PPAR-α, peroxisome proliferator-activated receptor-α; Bcl-xL, B-cell lymphoma-extra large.

2.2. Experimental procedures

Diabetic mice were divided into two nonischemic and six ischemic groups, with blood glucose levels as similar as possible among the groups. The baseline blood glucose levels of all groups were shown in Table 3. One of the nonischemic and three of the six ischemic groups were fed with a semipurified diet (comparable to AIN 93 M), while the other nonischemic and the remaining three ischemic groups received the intervention diet, which is identical to the control diet except that part of the total casein protein content was substituted with GLN. The control diet contained 19.6-g GLN/kg, but the GLN-supplemented diet contained 54.6-g GLN/kg. The experimental diet was made in our laboratory. Both diets were isocaloric and isonitrogenous in character (Table 1). Corn starch, casein, fiber, mineral mixture and vitamin mixture were purchased from MP Biochemicals, LLC (Santa Ana, CA, USA). GLN, L-cysteine, choline and tert-butylhydroquinone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sucrose and soybean oil were purchased from Taiwan Sugar Co. (Taipei, Taiwan). The dosage of GLN chosen had been used previously and was found to increase antioxidant potential and reduce inflammation in diabetic rodents [26,27]. Treated as the nonischemic control groups, mice were sacrificed after being fed with either the control or GLN-supplemented diet for 3 weeks before sacrificed. As for the ischemic groups, unilateral hindlimb ischemia was induced in the ischemic groups by excision of the left femoral artery [28]. Briefly, mice were deeply anesthetized with an intraperitoneal injection of Zoletil (25 mg/kg BW) and Rumpun (10 mg/kg BW). Proximal and distal portions of the femoral artery were exposed and ligated with silk sutures. Mice were given subcutaneous injection of buprenorphine (0.1-mg/kg BW) for pain control *via* a subcutaneous injection every 12 h for 2 days after surgery. The six ischemic groups were first divided into groups fed with either the control or GLN-

Table 3
Plasma glucose levels among the different groups at the baseline, beginning and end of the experiment.

Group	Baseline before diabetes *	Beginning, mg/dL	Final
DC	162.6±5.6	214.8±6.1	308.5±12.1[a]
DG	160.4±2.7	215.6±2.5	311.9±11.6[a]
IC1	156.8±7.2	217.0±3.6	252.0±16.0[b]
IG1	163.2±10.9	222.8±5.5	240.1±16.5[b]
IC7	158.6±6.5	222.0±6.9	368.5±27.2[a]
IG7	171.4±3.9	215.2±4.8	286.8±19.7[c]

NC, nonischemic control group; NG, nonischemic glutamine (GLN) group; IC1, ischemic control group sacrificed on day 1; IC7, ischemic control group sacrificed on day 7; IG1, ischemic GLN group sacrificed on day 1; IG7, ischemic GLN group sacrificed on day 7. All data are representative of duplicate measurements (n=8). Data are presented as the mean ± S.E.M. Differences among groups were analyzed by a two-way ANOVA with Bonferroni's post-hoc test. Values in the column without common letter are significant difference at P<.05.

* Significantly differ from the levels at beginning and end of the experiment after STZ injection.