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The Regulatory Role of the Hexosamine Biosynthesis Pathway in Vascular Endothelial Inflammation (Postprint)

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Abstract

Background Atherosclerosis (AS) is the main pathological basis of cardiovascular disease, characterized by vascular endothelial inflammation; therefore, targeting inflammation-related mechanisms is key to the prevention and treatment of AS. Objective To investigate the effects of the hexosamine biosynthesis pathway (HBP) on adhesion molecules and its regulatory role in vascular endothelial inflammation. Methods From August to December 2022, 24 SPF-grade female C57BL/6 mice were divided into control, 6-diazo-5-oxo-L-norleucine (DON), high-fat diet (HFD), and HFD+DON groups using a randomized block design method based on body weight. After 15 weeks of high-fat diet feeding and intraperitoneal DON injection, mouse serum and aortic tissues were collected. Biochemical assay kits were used to detect blood lipid levels before and after intervention, hematoxylin-eosin (HE) staining was employed to examine pathological changes in the aortic root, and immunofluorescence staining, ELISA, and Western blotting were performed to detect the expression levels of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Results After 15 weeks of intervention, compared with the control group, the HFD group showed significantly increased LDL-C and TC levels, while HDL-C was significantly decreased ($P<0.05$); there was no change in blood lipid levels between the HFD and HFD+DON groups. HE staining results revealed that the HFD group exhibited thickened vascular intima, abnormal vascular smooth muscle morphology, disorganized structure, and numerous foam cells. In the HFD+DON group, mouse smooth muscle cells were neatly arranged, the endothelial cell layer was continuous, the number of foam cells was significantly reduced, and intercellular spaces were essentially normal. Immunofluorescence staining, ELISA, and Western blotting results all showed that ICAM-1 and VCAM-1 protein expression was downregulated in the HFD+DON group compared with the HFD group. Conclusion Inhibition of

HBP has the effect of downregulating adhesion molecules ICAM-1 and VCAM-1 expression and ameliorating vascular endothelial inflammation.

Full Text

The Regulatory Role of Hexosamine Biosynthesis Pathway in Vascular Endothelial Inflammation

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Abstract

Background: Atherosclerosis (AS) is the main pathological basis of cardiovascular disease and is characterized by vascular endothelial inflammation, thus targeting inflammation-related mechanisms is the key to prevention and treatment of AS. **Objective:** To investigate the effect of the hexosamine biosynthesis pathway (HBP) on adhesion molecules and its regulatory role in vascular endothelial inflammation. **Methods:** From August to December 2022, 24 SPF grade C57BL/6 female mice were divided into control group, DON group, HFD group, and HFD+DON group according to randomized block design method using body weight stratification. Serum and aortic tissue from the mice were collected after 15 weeks of administration of high-fat diet and intraperitoneal injection of DON. The lipid levels of mice were detected using biochemical kits before and after intervention, pathological changes in the aortic root were detected by HE staining, and the expression levels of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were detected by immunofluorescence staining, ELISA and Western blot. **Results:** After 15 weeks of intervention, compared with the control group, the levels of LDL-C and TC were increased significantly in the HFD group, while HDL-C was reduced significantly ($P<0.05$); there was no change in the lipid levels between the HFD group and the HFD+DON group. HE staining results showed that the vascular intima was thickened, the morphology of vascular smooth muscle was abnormal, the structure was disorganized, and a large number of foam cells were seen in HFD group. The smooth muscle cells of mice were neatly aligned, the endothelial cell layer was continuous, the number of foam cells was reduced significantly, and the cell gap was basically normal in the HFD+DON group. The results of immunofluorescence staining, ELISA and Western blot showed that the expression of ICAM-1 and VCAM-1 was down-regulated in the HFD+DON group compared with the HFD group. **Conclusion:** Inhibition of HBP can down-regulate the expression of ICAM-1 and VCAM-1, and play a role in improving

vascular endothelial inflammation.

Key words: Vascular endothelial inflammation; Hexosamine biosynthetic pathway; Intercellular adhesion molecule-1; Vascular cell adhesion molecule-1; 6-diazo-5-oxo-L-norleucine; Mice

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Introduction

With population aging, the incidence of cardiovascular disease in China has risen rapidly, with mortality remaining at the top of the list [1]. Under the influence of multiple risk factors such as hyperlipidemia [2-3], hyperglycemia [4], and hypertension [5], vascular endothelial injury occurs, thereby increasing the risk of atherosclerotic cardiovascular disease. Damaged endothelial cells secrete various adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [6], which subsequently enhance leukocyte permeability and promote foam cell accumulation in the vascular wall, ultimately leading to the initiation and progression of atherosclerotic inflammation [7-8]. Therefore, regulating the expression of adhesion molecules may be key to preventing and treating atherosclerotic inflammation.

Adhesion molecules ICAM-1 and VCAM-1 are transmembrane proteins belonging to the immunoglobulin superfamily that require extensive glycosylation modification to execute their pro-inflammatory functions [9-10]. Under the action of key enzymes in the hexosamine biosynthesis pathway (HBP), fructose-6-phosphate and glutamine are catalyzed to form glucosamine-6-phosphate, ultimately generating the main donor substrate for glycosylation, uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) [11-13]. When energy demand surges, HBP becomes overactivated, leading to elevated UDP-GlcNAc levels and enhanced glycosylation effects [14-16]. Moreover, enhanced glycosylation can upregulate ICAM-1 and VCAM-1 expression, promoting inflammation initiation and progression [17-18]. Therefore, elucidating how HBP regulates adhesion molecule glycosylation modification may be important for improving vascular endothelial inflammation.

6-diazo-5-oxo-L-norleucine (DON) is a glutamine antagonist that competitively binds to glutamine sites on HBP, reducing UDP-GlcNAc levels [19-21]. In vitro experiments have demonstrated that targeting glutamine metabolism through DON to inhibit HBP can downregulate VCAM-1 transcription levels in mesangial cells and improve vascular endothelial cell inflammation [22]. However, in vivo experimental validation of HBP's role in vascular endothelial inflammation has not been reported to date. Therefore, this study aims to further investigate the effect of HBP on adhesion molecules and its regulatory role in vascular endothelial inflammation at the animal level, providing a basis for preventing and treating atherosclerotic inflammation.

Materials and Methods

Experimental Animals and Feed Twenty-four SPF-grade female C57BL/6 mice, aged 7 weeks and weighing 14.3–17.9 g, were purchased from the Laboratory Animal Center of Guangxi Medical University (certificate number: SYXK Gui 2020-0004) and housed in the animal facility of the School of Public Health at Guangxi Medical University. The housing conditions were maintained at 20–26°C with 40–70% relative humidity and a 12-hour light/dark cycle. Mice were fed in individually ventilated cages with free access to food and water. The high-fat diet (composition: 15% fat + 1.25% cholesterol + 0.5% bile salts + 83.25% basal feed) was purchased from Nantong Trophic Animal Feed High-Tech Co., Ltd. The experiment was approved by the Laboratory Animal Ethics Committee of Guangxi Medical University (approval number: 202201191).

Main Reagents and Instruments The following reagents and instruments were used: DON (GLPBIO, catalog number: GC41224); low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) assay kits (Nanjing Jiancheng Bioengineering Institute, catalog numbers: A113-1-1, A111-1-1, A112-1-1); ICAM-1 and VCAM-1 enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Aimeng Youning Biotechnology Co., Ltd., catalog numbers: LV30245M, LV30555M); bicinchoninic acid (BCA) protein concentration assay kit, sodium dodecyl sulfate polyacrylamide gel electrophoresis rapid preparation kit, and radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, catalog numbers: P0010, P0012AC, P0013B); hematoxylin-eosin (HE) dye (Servicebio, catalog number: G1003); ICAM-1 antibody and VCAM-1 antibody (Abcam, catalog numbers: ab171123, ab134047); β-actin (CST, catalog number: 4970S). Equipment included a multifunctional microplate reader (ThermoFisher, model: Multiskan GO), paraffin microtome (Leica, model: RM2016), fluorescence microscope (Nikon, model: Eclipse C1), intelligent gel imaging system (ThermoFisher, model: iBright FL1000), and high-speed low-tissue grinder (Servicebio, model: KZ- -F).

Animal Grouping and Administration After acclimation, mice were divided into four groups (n=6 each) using a randomized block design based on body weight: control group, DON group, high-fat diet (HFD) group, and HFD+DON group. The control group received regular chow and intraperitoneal injection of phosphate-buffered saline (PBS) (1 mg/kg, 3 times/week). The DON group received regular chow and intraperitoneal injection of DON solution (1 mg/kg, 3 times/week). The HFD group received high-fat diet and intraperitoneal injection of PBS (1 mg/kg, 3 times/week). The HFD+DON group received high-fat diet and intraperitoneal injection of DON solution (1 mg/kg, 3 times/week). After 15 weeks of intervention, mice were anesthetized with avertin and blood was collected via the orbital sinus. Blood was allowed to clot for 2 hours, then centrifuged at 3,000 rpm for 10 minutes, and serum was stored at -20°C. After blood collection, mice were euthanized and perfused systemically with pre-cooled PBS and paraformaldehyde until the liver turned

from red to white, indicating complete perfusion. Aortic tissues were harvested, with some fixed in paraformaldehyde for 48 hours and others stored in sterile cryovials at -20°C.

Serum Lipid Level Measurement Before intervention and after 15 weeks of intervention, blood was collected from the submandibular vein after overnight fasting with free access to water. Blood was allowed to clot for 2 hours, then centrifuged at 3,000 rpm for 10 minutes to collect serum. Levels of LDL-C, TC, and HDL-C were measured using biochemical assay kits.

HE Staining Aortic tissues fixed in 4% paraformaldehyde were processed into paraffin sections. Sections were sequentially placed in xylene I for 20 minutes, xylene II for 20 minutes, absolute ethanol I for 5 minutes, absolute ethanol II for 5 minutes, and 75% ethanol for 5 minutes for deparaffinization and hydration. After deparaffinization, sections were stained with hematoxylin for 3–5 minutes, rinsed, differentiated in differentiation solution, rinsed again, treated with bluing solution, and rinsed. Sections were then dehydrated sequentially in 85% and 95% ethanol for 5 minutes each, stained with eosin for 5 minutes, and further dehydrated in absolute ethanol I, II, and III for 5 minutes each, followed by xylene I and II for 5 minutes each. Finally, sections were mounted with neutral balsam and examined under a microscope.

Immunofluorescence Staining and Observation Embedded specimens were sectioned into paraffin slices. After deparaffinization and hydration, antigen retrieval was performed. Slides were placed in PBS (pH=7.4) and washed on a decolorizing shaker three times for 5 minutes each. Sections were dried, and a histochemical pen was used to outline the tissue. Bovine serum albumin was applied for blocking for 30 minutes. Primary antibodies were added, and sections were incubated overnight at 4°C in a humidified chamber. After primary antibody incubation, slides were washed. Corresponding secondary antibodies were applied and incubated at room temperature in the dark for 50 minutes. After secondary antibody incubation, slides were washed again. 4',6-diamidino-2-phenylindole (DAPI) staining solution was added and incubated at room temperature in the dark for 10 minutes. After staining, slides were washed again. Autofluorescence quencher solution B was applied for 5 minutes, followed by a 10-minute rinse, and sections were mounted with anti-fluorescence quenching mounting medium. Images were captured using a fluorescence microscope in the dark.

ELISA Detection Serum samples were collected from mice, and serum ICAM-1 and VCAM-1 levels were measured using ELISA according to the manufacturer's instructions.

Western Blot Detection Aortic tissue samples were collected and ground, then protein was extracted using RIPA lysis buffer. Protein concentration was

determined using the BCA method. Protein loading buffer was added, and samples were subjected to electrophoresis and transferred to membranes. Polyvinylidene fluoride membranes were blocked in 5% skim milk at room temperature for 2 hours. Primary antibodies against ICAM-1, VCAM-1, and β-actin were added and incubated overnight at 4°C. Membranes were washed three times with Tris-buffered saline containing Tween-20 for 10 minutes each. Corresponding secondary antibodies were added and incubated at room temperature on a shaker for 1 hour, followed by thorough washing to remove excess secondary antibody. Membranes were scanned using an intelligent gel imaging system, and band intensities were analyzed using Image J software. All target proteins were normalized to β-actin as an internal reference.

Statistical Analysis Statistical analysis was performed using STATA 17.0 and SPSS 25.0 software. Measurement data are expressed as mean $\pm$ standard deviation ($\bar{x}\pm s$). Comparisons among multiple groups were performed using one-way ANOVA, pairwise comparisons between groups were performed using LSD-t test, and comparisons before and after intervention within the same group were performed using paired t-test. $P<0.05$ was considered statistically significant.

Results

Serum Lipid Levels in the Four Groups Before intervention, there were no significant differences in serum LDL-C, TC, and HDL-C levels among the four groups ($P>0.05$). After 15 weeks of intervention, significant differences were observed in serum LDL-C, TC, and HDL-C levels among the four groups ($P<0.05$). Specifically, LDL-C and TC levels in the HFD and HFD+DON groups were higher than those in the control and DON groups, while HDL-C levels were lower than those in the control and DON groups ($P<0.05$). Compared with pre-intervention values, serum LDL-C and TC levels in all four groups increased after 15 weeks of intervention, while HDL-C levels decreased in the control, HFD, and HFD+DON groups ($P<0.05$). The results are shown in Table 1 .

HE Staining of Aortic Root Cross-Sections HE staining results showed that aortic smooth muscle morphology was normal with orderly arrangement and intact endothelial structure in both the control and DON groups, with no inflammation observed (Figure 1 [Figure 1: see original paper]A, 1B). In the HFD group, the vascular intima was thickened, vascular smooth muscle morphology was abnormal with disordered arrangement, and numerous foam cells were visible (Figure 1C). In the HFD+DON group, smooth muscle cells were relatively orderly arranged, the endothelial cell layer was continuous, the number of foam cells was significantly reduced, and cell gaps were essentially normal (Figure 1D).

Immunofluorescence Staining of Aortic Root Cross-Sections Immunofluorescence staining results showed that compared with the control

group, ICAM-1 and VCAM-1 expression in the aortic root cross-sections showed no obvious changes in the DON group but was significantly increased in the HFD group. Compared with the HFD group, ICAM-1 and VCAM-1 expression was reduced in the HFD+DON group. Representative images are shown in Figure 2 [Figure 2: see original paper] and Figure 3 [Figure 3: see original paper].

Serum Adhesion Molecule ICAM-1 and VCAM-1 Levels Significant differences were observed in serum ICAM-1 and VCAM-1 levels among the four groups ($P<0.05$). Specifically, serum ICAM-1 level in the HFD+DON group was lower than that in the control, DON, and HFD groups, while serum ICAM-1 level in the HFD group was higher than that in the DON group ($P<0.05$). Serum VCAM-1 level in the HFD+DON group was lower than that in the DON and HFD groups, while serum VCAM-1 level in the HFD group was higher than that in the control group ($P<0.05$). The results are shown in Table 2 .

Protein Expression of ICAM-1 and VCAM-1 in Aortic Tissues There was no significant difference in ICAM-1 protein expression among the four groups ($P>0.05$). However, significant differences were observed in VCAM-1 protein expression among the four groups ($P<0.05$). Specifically, VCAM-1 protein expression in the HFD group was higher than that in the control, DON, and HFD+DON groups ($P<0.05$). The results are shown in Table 3 and Figure 4 [Figure 4: see original paper].

Discussion

HBP serves as a sensor of metabolic flux and a precursor for glycosylation modifications [23-24]. The interaction between advanced glycation end products and their receptors can trigger cell activation [25]. Once vascular endothelial cells are activated, they induce increased expression of adhesion molecules ICAM-1 and VCAM-1, leading to immune cell adhesion and vascular wall inflammation [26]. Therefore, investigating the regulatory role of HBP in vascular endothelial inflammation is crucial. Studies have shown that adipocyte hypertrophy in obesity leads to decreased glutamine levels, altering energy metabolism and HBP activity, with upregulated glycosylation modification levels ultimately increasing pro-inflammatory gene transcriptional activity [27]. LIN et al. [28] found that inhibiting HBP with DON in human lung adenocarcinoma cells reduced intracellular UDP-GlcNAc levels and decreased glycosylation effects. JAMES et al. [22] further demonstrated at the cellular level that inhibiting key HBP enzyme activity could downregulate VCAM-1 transcription levels in mesangial cells and improve cellular inflammation. Based on animal experiments, our results show that HFD induced inflammatory responses in the vascular intima, while inhibiting HBP downregulated adhesion molecule ICAM-1 and VCAM-1 expression, indicating that HBP plays an important role in improving vascular endothelial inflammation.

Dyslipidemia and vascular endothelial inflammation are not independent of each other and can both accelerate atherosclerosis progression [29-31]. Serum TC, LDL-C, and HDL-C levels are major indicators reflecting lipid metabolism [32]. YU et al. [33] showed that HFD-induced lipid metabolism imbalance leads to elevated serum LDL-C and TC levels and decreased HDL-C levels, enhancing oxidative stress and inflammatory injury in mice. Our results demonstrate that after 15 weeks of intervention, LDL-C and TC levels in the HFD and HFD+DON groups were higher than those in the control and DON groups, while HDL-C levels were lower, indicating that a hyperlipidemic state had been established. These findings are consistent with WANG et al. [34], confirming that HFD can induce lipid metabolism disorders and inflammatory responses in mice. Meanwhile, our results show that DON did not alter lipid levels, suggesting that the anti-vascular endothelial inflammatory effect of HBP inhibition is independent of changes in lipid levels.

Foam cells formed by excessive cholesterol accumulation in macrophages are a pathological hallmark of atherosclerosis [35-36]. Our study found that smooth muscle morphology was normal with intact structure and orderly arrangement in both the control and DON groups, with no inflammation observed. In contrast, the HFD group showed thickened vascular intima, abnormal smooth muscle cell morphology, disordered structure and arrangement, numerous foam cells, and inflammatory cell infiltration. These results largely align with VIKRAM et al. [37], suggesting that HFD activates vascular intimal inflammatory responses. In the HFD+DON group, abnormal aortic structure was improved, the endothelial cell layer was continuous, and foam cells were significantly reduced. These findings indicate that under normal conditions, inhibiting HBP does not alter vascular morphology, but when HFD induces vascular intimal inflammatory responses, HBP inhibition can exert anti-inflammatory effects.

The early stage of atherosclerosis is closely associated with inflammatory changes in vascular endothelial cells. When endothelial cells are damaged, adhesion molecules such as ICAM-1, VCAM-1, and E-selectin are released, inducing lymphocyte and monocyte infiltration into the arterial wall and promoting inflammation development [38-39]. Furthermore, glycosylation modifications play an important role in adhesion molecule recognition and cell-cell interactions during early atherosclerotic plaque formation [40]. Studies have shown that targeting glutamine metabolism with DON to inhibit HBP can reduce intracellular UDP-GlcNAc levels, thereby affecting intracellular protein glycosylation modification processes [41-42]. PARK et al. [43] also found that DON could inhibit growth factor-stimulated proliferation and migration of vascular smooth muscle cells and improve atherosclerotic restenosis. Our study found that under normal vascular endothelial conditions, DON intervention did not change ICAM-1 and VCAM-1 expression. However, when HFD induced vascular intimal inflammatory responses and upregulated adhesion molecule expression, DON intervention significantly reduced ICAM-1 and VCAM-1 expression in mouse serum, and ICAM-1 and VCAM-1 protein expression in arterial tissues was lower than that in the HFD group. Meanwhile, immunofluorescence staining of aortic root cross-

sections also detected lower ICAM-1 and VCAM-1 positive expression than in the HFD group. These results suggest that under inflammatory conditions, inhibiting HBP can improve HFD-induced vascular endothelial inflammation in mice.

In summary, this study investigated the intrinsic pro-inflammatory mechanism in endothelium—that HBP can regulate the transmembrane expression of adhesion molecules ICAM-1 and VCAM-1—and validated the regulatory role of HBP in improving vascular endothelial inflammation using in vivo experiments, providing new insights for preventing and treating early pathological progression of cardiovascular disease. However, this study did not measure glycosylation metabolites associated with HBP-related adhesion molecules. Therefore, whether the regulatory role of HBP is related to protein glycosylation modification requires further investigation in subsequent work.

Author Contributions

CHEN Yijing conceptualized and designed the study, implemented the research, and wrote the manuscript. XU Qi and LIU Zhongdian collected and organized data, performed statistical analysis, and prepared figures and tables. CHEN Yijing and CHEN Shuping conducted experimental measurements. QIN Lingqiao and TANG Weiting revised the manuscript. ZHONG Qiuan was responsible for quality control and review of the article, overall supervision, and management.

Conflict of Interest

The authors declare no conflict of interest.

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