High-fat diet up-regulates caveolin-1 expression in aorta of diet-induced obese but not in diet-resistant rats

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Abstract

Objective: Caveolin-1 (Cav-1) is considered as a negative regulator of endothelial nitric oxide synthase (eNOS) and influences various cardiovascular functions. The objective of the study is to investigate the effects of high-fat diet on vascular Cav-1 expression and eNOS activities.

Methods: Male outbred Sprague-Dawley rats were fed with a high-fat diet for 15 weeks to induce obesity. The diet-induced obese (DIO) rats were then divided into two groups. One group (DIO-LF) was fed a normal diet and another group (DIO-HF) along with diet-resistance (DR) rats were fed a high-fat diet for 8 more weeks. Cav-1 expression was determined using reverse transcription (RT)-PCR, Western blotting, and immunohistochemistry techniques. NOS activities were assessed using Griess reagents. Protein kinase B (PKB/Akt) and eNOS expression and phosphorylation were determined by Western blotting.

Results: A high-fat diet up-regulated Cav-1 and down-regulated eNOS expression in aorta of DIO rats, but not in that of DR rats. These effects were reversed in DIO rats after switching to a low-fat diet for 8 weeks. Decreased NOS activities in aortas were observed in DIO rats, but not in DR rats. Phosphorylation of PKB/Akt and eNOS (Ser1177) were enhanced in aortas of DIO rats of both DIO-HF and DIO-LF groups.

Conclusion: These findings suggest that the decrease of vascular NOS activities in rats fed a high-fat diet were due, at least in part, to the up-regulation of Cav-1 expression.

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Keywords: High-fat diet; Obesity; Diet-resistance; Caveolin-1; Endothelial nitric oxide synthase

1. Introduction

Obesity is one of the most important risk factors contributing to cardiovascular diseases [1]. However, the underlying mechanisms are not well understood. Many studies have demonstrated that excess weight is possibly associated with impaired endothelial function and proposed that metabolic and inflammatory responses to the increased amount of fat storage may be involved [2]. This impairment of endothelial function becomes obvious early on, long before any vascular function becomes clinically relevant and detectable [3].

The healthy endothelium is maintained in homeostatic balance by a series of anti-inflammatory, antithrombotic, profibrinolytic, and vasodilatory functions. Endothelium-derived nitric oxide (NO) plays a pivotal role in these functions. In endothelia, NO is synthesized by endothelial NO synthase (eNOS) which can be mediated by many physiopathological factors such as shear stress and hypercholesterolemia or metabolites such as glucose and free fatty acids in vitro [4–7].

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Although the impairment of NO production is considered to be at least one of the causes of endothelial dysfunction, the regulations of eNOS expression and activities are complex and not fully understood. At basal state, the majority of eNOS appears to be bound to Cav-1, the primary coat protein and structural component of caveolae, with its active site repressed in caveolae that consist of uniform 50- to 100-nm invaginations of the plasma membrane [8]. Insights into the roles of caveolae from Cav-1 knock-out mice suggest that Cav-1 may be profoundly important for postnatal cardiovascular functions, including endothelial barrier function, regulation of NO synthesis, cholesterol metabolism, and cardiac functions [9]. It is considered that Cav-1 negatively regulates eNOS activities via protein–protein interactions [10,11].

A previous report showed that caveolin-deficient mice were lean and resistant to diet-induced obesity [12]. Another report by Cohen et al. [13] further indicated that a defect in insulin-regulated lipogenesis was responsible for the lean body phenotype. However, to our knowledge, no study has yet been conducted in non-Cav-1 deficient animals to address whether high-fat diet-induced obesity relates to Cav-1 expression in the vascular system. The objective of this study was to investigate the effects of a high-fat diet on vascular Cav-1 expression and eNOS activities.

2. Methods

2.1. Animals and experimental protocol

A diet-induced obesity model, modified from Kowalski’s report [14], was made. Briefly, 36 male Sprague-Dawley rats weighing about 160–170 g were housed individually in cages under controlled conditions (a 12-h light/dark cycle; 17–27 °C; 40–60% relative humidity). 27 animals were fed with a high-fat diet containing 19.33 kJ/g (49.85% fat, 20% protein and 30.15% carbohydrate) and 9 rats (control group) were fed with normal laboratory chow containing 13.77 kJ/g (13.68% fat, 21.88% protein and 64.44% carbohydrate). Animals had ad libitum access to food and water. After 15 weeks, rats with the highest weight gain (DIO, n = 14) and the lowest weight gain (DR, n = 7) were selected for further study. The DIO rats were then randomly subdivided into two groups: 7 rats were shifted to normal chow diet with lower fat (DIO-LF) and the other 7 rats continued to be fed with high-fat diet (DIO-HF). At this stage, there were four groups of rats in our experiments, namely, the control (n = 9), the DIO-LF (n = 7), the DIO-HF (n = 7), and the DR (n = 7) groups. The experiment was continued for another eight weeks. At the end of the experiment, all rats were sacrificed after 12 h fasting. Thoracic aorta and other tissues were rapidly separated, immediately frozen in liquid nitrogen, and then stored at −80 °C until analysis. All experiments were conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare. The investigation also confirmed with the Guided for the Care and Use of Laboratory Animals (NIH Publication No.85-23, revised 1996).

2.2. Analysis of blood samples

Blood was collected from the tail vein between 8:00 AM and noon under overnight fasting conditions during the experimental period. All serum samples were stored at −80 °C until analysis. Serum glucose concentration was determined using the glucose oxidase method [15]. Serum total cholesterol and triglycerides were measured by CHOD-PAP and GPO-PAP methods, respectively.

2.3. NOS activities

Activity of NOS was determined by Griess reactions [16]. Briefly, the total NOS (tNOS) activity was assayed by adding the supernatant of aortic homogenate to the incubation buffer containing Bis–Tris propane (pH 7.2), L-arginine, DTT, NADPH, tetrahydro–l-biopterin and CaCl2. NADPH was removed with lactate dehydrogenase and sodium pyruvate at the end of reaction. Nitrite levels were determined using Griess reagents. The activity of inducible NOS (iNOS) in arterial vascular was further characterized by incubation with EDTA instead of CaCl2. The constitutive NOS (cNOS) activity was calculated by subtract iNOS from tNOS activity.

2.4. Electrophoresis and immunoblotting

Aortic segments were homogenized in extraction buffer and incubated on ice for 2 h. Electrophoresis and immunoblotting were employed as described previously [17]. Rabbit anti-caveolin-1 polyclonal antibody, mouse anti-Akt1 monoclonal antibody, and rabbit anti-p-Akt1/2/3 polyclonal antibody were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Endothelial nitric oxide synthase expressions and phosphorylations were detected with rabbit anti-endothelial nitric oxide synthase polyclonal antibody (Cayman Chemical Company, Ann Arbor, MI) and rabbit anti Ser1177 p–eNOS polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Mouse anti-α-tubulin (Sigma, Saint Louis, MO) was used as a control. Immunoreactive bands were detected by means of an ECL plus Western blot Detection System (Amersham Biosciences, Buckinghamshire, England) and quantified with a Biometra densitometer (Biometra, Goettingen, German).

2.5. Reverse transcription (RT)-PCR

Total RNA was extracted from tissues following a modification of the guanidinium thiocyanate method [18]. Reverse transcription was carried out with 2.5 μg mRNA in 25 μl containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 50 mM dithiothreitol, 20 U of RNase inhibitor (Biostar International, Canada) and 200 U of M-MLV reverse transcriptase (Promega corporation, Madison, USA). PCR was performed in a total volume of 50 μl containing 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 200 μM dNTPs, 100 ng of the appropriate primers (caveolin 5'-primer, TCT
ACA AGC CCA ACA ACA AGG; and caveolin 3′-primer, AGG AAA GAG AGG ATG GCA AAG, the expected size of the amplicon is 304 bp. β-actin 5′-primer, CAT CAC TAT CGG CAATGA GC; and β-actin 3′-primer, GAC AGC ACT GTG TTG GCA TA, the expected size of the amplicon is 156 bp), 1.5 μl of reverse-transcribed cDNA, and 4.8 U of Taq polymerase (Biostar International, Canada). The reaction was amplified with a DNA thermal cycler (Biometra, Goettingen, Germany) for 30 cycles. The amplification profile involved denaturation at 95 °C for 1 min, primer annealing at 55 °C for 40 s, and elongation at 72 °C for 40 s. 10 μl of each PCR reaction was mixed with 2 μl of 6-fold concentrated loading buffer and loaded onto a 1.5% agarose gel containing ethidium bromide. Electrophoresis was carried out at a constant voltage of 100 V for 30 min and quantified with a Biometra densitometer (Biometra, Goettingen, German).

2.6. Immunohistochemistry

Streptavidin-biotin-peroxidase complex staining was employed as described previously [19]. Rat aortic segments were isolated and the frozen sections (8 μm in thickness) were thaw-mounted onto precleaned glass slides and kept overnight in a desiccator at 4 °C. After washing in phosphate-buffered saline and fixation in acetone, slides were blocked with 3% H2O2 and then blocked with 10% normal goat serum for 30 min at room temperature. The slices were then incubated with rabbit anti-caveolin-1 overnight at 4 °C and with goat anti-rabbit biotin IgG for 30 min at room temperature. After reacted with spereptavidin-biotin-peroxidase complex for 30 min, immunoreactivity was determined by 3,3′-diamino benzidinetetrahydrochloride solution. To better appreciate negative nuclei, the slices were counterstained with hematoxylin.

2.7. Statistical analysis

Data were expressed as mean±SEM. Experiments of protein and mRNA expression were reproduced three to four times and the representative experiments are shown. Data were analyzed by either one-way ANOVA or repeated measures ANOVA and multiple comparisons were done by LSD (least significant difference) t-test. Differences were considered significant when p<0.05.

3. Results

3.1. Effects of high-fat diet on rat body weight and visceral fat deposits

The ANOVA and the tests of between-subjects effects showed significant differences in weight gain among the four experimental groups (F=18.846, p=0.000, Fig. 1 A). The pairwise comparisons based on estimated marginal means showed that the mean difference was significant (p<0.05) and the mean body weight of DIO rats (DIO-HF and DIO-LF) increased significantly compared with the control rats (p<0.01) or DR rats (p<0.01). During the diet-inducing period (first 15 weeks), the DIO rats gained more body weight than DR rats and normal chow-fed control rats. The significant differences between DIO rats and control (p<0.05) began from the 7th week and persisted at the end of the study even though DIO rats were shifted to normal chow from the 15th week (DIO-LF rats, p<0.05 comparing with the control rats). Changes in mean body weight showed that there was no difference between DR and control rats during the whole experimental period despite substantial differences in their diet constituents. At the end of this study, the mean body weight of DIO-LF rats was lower than that of DIO-HF rats (p<0.05) and higher than that of DR rats (p<0.05).

Fig. 1. Effects of high-fat diet on body weight in male Sprague-Dawley rats. The high-fat diet-fed rats were divided into three groups, namely, DIO-LF (n=7), DIO-HF (n=7), and DR (n=7) at the end of 15th week according to the protocol in Methods section. (A) Body weights were measured weekly and the data were presented as means±SEM. Symbol (*) represents significant differences comparing with the control group (n=9). Different letters (a, b) attached to DIO-LF group represent significant differences comparing with DIO-HF and DR group, respectively (p<0.05). (B) White adipose tissue from visceral fat deposits was measured and wet weight was presented as means±SEM. *p<0.05 compared with the bars indicated. DIO-LF, high-fat diet induced obese rats shift back to lower fat normal chow diet; DIO-HF, high-fat diet induced obese rats continued to be fed with high-fat diet; DR, high-fat diet resistance rats.
The visceral fat deposits of DIO-HF rats at the end of study increased significantly compared with the control \( (p<0.05) \), DIO-LF \( (p<0.05) \) and DR \( (p<0.05) \) rats, respectively (Fig. 1B) whereas no significant difference of visceral fat deposits was found among DIO-LF, DR and the control rats.

3.2. Effects of high-fat diet on serum glucose and lipid concentrations

As shown in Fig. 2A, high-fat diet increased the serum glucose concentrations significantly in both DIO and DR rats at the 15th week, the end of diet inducing period \( (p<0.05) \),
and at the 23rd week comparing with normal diet rats \((p<0.05)\). Switching to normal diet resulted in decrease in serum glucose (DIO-LF versus control, \(p>0.05\)). There was a tendency toward increasing serum cholesterol concentrations in DIO rats but not in DR rats fed with high-fat diet. However, no significant difference in serum cholesterol concentration was observed between DIO and normal diet control rats (Fig. 2B). Serum triglyceride concentrations were significantly increased \((p<0.05)\) in DIO rats but not in DR rats compared to normal diet control rats (Fig. 2C). After being shifted to normal chow for 8 weeks, no difference of serum triglyceride concentration was observed between DIO-LF and control rats \((p>0.05)\).

3.3. NOS activities in thoracic aorta

NOS activities were measured in the aorta of high-fat fed DIO, DR, and normal diet control rats \((n=5)\). Both total NOS activities and constitutive NOS decreased significantly in DIO rats comparing with DR or control rats \((p<0.05)\). No difference was observed between DR and control rats (Fig. 3).

3.4. Cav-1 mRNA and protein expression in thoracic aorta

Cav-1 and caveolae play important roles in the activities of eNOS and cholesterol transport. Stimuli that increase or decrease Cav-1 levels regulate the number of caveolae and thus the eNOS activities. The effect of high-fat diet on Cav-1 expression at mRNA and protein levels was therefore examined. As shown in Fig. 4A and B, high-fat diet up-regulated the expression levels of Cav-1 mRNA and protein in DIO-HF rats, but not in DR rats. The elevation of Cav-1 mRNA and protein levels was diminished in DIO rats fed with normal chow diet for 8 weeks (DIO-LF vs. DIO-HF, \(p<0.05\)).

3.5. Cav-1 immunohistochemistry analysis in thoracic aorta

The distribution of Cav-1 in thoracic aorta was further assessed using immunohistochemistry analysis. As shown in Fig. 5, immunoreactivity of Cav-1 was widely distributed in rat aorta from all groups. The stains of DIO-HF rats were much deeper than the other groups. This indicated that high-fat diet induced the over expression of Cav-1 protein in aorta. The results were also consistent with the data from Western blot and RT-PCR.

3.6. eNOS expression, eNOS (Ser 1177) and Akt1 (Ser473) phosphorylations in thoracic aorta

The effects of high-fat diet on the expression and phosphorylation of eNOS in thoracic aorta were then examined. The results of Western blot showed that high-fat diet down-regulated eNOS expression in DIO rats but not in DR rats (Fig. 6A, DIO-HF vs. Control, \(p<0.05\)). This effect diminished in DIO rats after being shifted to normal chow diet for 8 weeks (DIO-LF vs. DIO-HF, \(p<0.05\) and DIF-LF vs. Control, \(p>0.05\)). The phosphorylation levels of both eNOS (Ser 1177) and Akt1 (Ser473) in thoracic aorta were

![Immunohistochemistry for Cav-1 in rat aorta. Transversal sections of thoracic aorta from the control, DIO-LF, DIO-HF and DR rats were immunolabeled with antibodies against Cav-1 and biotinylated secondary antibodies as described in Methods section. 3',3'-diamino-benzidinetetrahydrochloride was used to locate Cav-1. Magnifications of indicated length are shown in each picture. The results are representative of three separate experiments.](image-url)
increased in DIO groups (Fig. 6B, both DIO-LF and DIO-HF vs. Control).

4. Discussion

The principal findings of this study were as follows: (1) high-fat diet up-regulated Cav-1 expression, down-regulated eNOS expression, and decreased NOS activities in rat thoracic aorta; (2) shifting to normal diet restored Cav-1 and eNOS levels; (3) no change in Cav-1 or eNOS expression was observed in DR rats fed with high-fat diet; (4) high-fat diet enhanced eNOS (Ser 1177) and Akt (Ser473) phosphorylation in diet-induced obese rats. In this study, we observed consistent elevations in aortic Cav-1 mRNA and protein levels in DIO rats. The induced Cav-1 abundance from high-fat diet was also shown by immunohistochemistry distribution. The decrease of both total NOS and constitutive NOS activities in DIO rats provided further evidence that the up-regulation of Cav-1 by high-fat diet inhibited cNOS (mainly eNOS) activities in aorta. In contrast to DIO rats, no changes in Cav-1 level, eNOS expression, and NOS activities were shown in DR rats even when they were fed with high-fat diets. And no changes of serum indices except glucose in DR rats were observed. These results suggested that the high-fat diet was involved in the regulation of Cav-1 and genetic background also played an important role. Previous studies have reported enhanced Cav-1 expression in male porcine coronary arteries fed with a high-fat diet [31] and no effect on Cav-1 expression in aorta of female Fischer rats [32]. In our study, shifting to normal chow restored the increased Cav-1 protein levels in DIO rats. This result not only provided further evidence of the effects of high-fat diets on aortic Cav-1 expression but also indicated the beneficial role of a low-fat diet.

Besides the effect of Cav-1 regulation, eNOS expression and PI3-K/Akt signalling pathway stimulation and its downstream eNOS phosphorylation (ser 1177) are also crucial to the production of NO. Furthermore, Molnar et al. [33]...
reported disrupted eNOS dimer formation rather than impaired insulin-mediated eNOS phosphorylation contributed to the endothelial dysfunction in diet-induced obese/diabetic mice. Their Western blot result showed that lower levels of eNOS expression were observed in aortas of mice fed with a high-fat diet. In our study, high-fat diet consumption reduced aortic eNOS expression in DIO rats which was restored by a low-fat diet, while enhanced protein kinase B (Akt) and eNOS phosphorylation was not restored by shifting to a low-fat diet. These results indicate that the regulation of NO production by a high-fat diet may be quite complex and several pathways may exist simultaneously.

In summary, the enhanced Cav-1 expression in aorta induced by a high-fat diet may be responsible for the increased risk of cardiovascular diseases in obese subjects, at least in part, via negatively regulating eNOS activities.

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