Anti-Oxidative Effect of Apocynin on Insulin Resistance in High-Fat Diet Mice

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Abstract. The present study examines the effects of apocynin on oxidative stress and antioxidant enzymes in high-fat diet (HFD) induced obese mice. After 12 weeks on HFD, the C57BL/6J mice that clearly exhibited insulin resistance received apocynin (2.4g/L) in their drinking water for five weeks. The results show that apocynin treatment significantly ameliorated hyperglycemia, hyperinsulinemia and dyslipidemia in HFD mice. Furthermore, the intraperitoneal glucose tolerance test (IPGTT) and homeostasis model assessment of insulin resistance (HOMA-IR) indicate significant improvement of insulin sensitivity in HFD mice after apocynin treatment. Compared to the HFD control mice, serum malondialdehyde (MDA) was significantly lower and serum superoxide dismutase (SOD) was significantly higher in apocynin treated HFD mice, indicating that apocynin suppressed systemic oxidative stress in the treated group. In the liver, apocynin significantly reduced the level of MDA. Accordingly, apocynin treatment strengthened the antioxidative defense system with an increased activity of SOD, glutathione-peroxidase (GSHpx) and content of reduced glutathione (GSH). We also found that hepatic catalase (CAT) activity significantly decreased after apocynin treatment which may indicate that apocynin reduces hydrogen peroxide and oxidative stress in the liver. These results suggest that apocynin may ameliorate insulin resistance by reducing systemic and hepatic oxidative stress in HFD fed mice.

Key words: apocynin, oxidative stress, insulin resistance, high-fat diet

Introduction

Insulin resistance syndrome, otherwise known as metabolic syndrome, is defined by the presence of insulin resistance, hyperglycemia, hyperinsulinemia, and some combination of obesity, dyslipidemia, inflammation and endothelial dysfunction [1, 2]. Consequently, insulin resistance syndrome has emerged as a major cause of type 2 diabetes.

It is now accepted that oxidative stress plays a critical role in the pathogenesis of insulin resistance [3]. Previous animal studies have demonstrated that chronic consumption of a high-fat diet (HFD) induces obesity, hyperglycemia, hyperinsulinemia, dyslipidemia and insulin resistance in C57BL/6J mice [4-6]. It is noteworthy that increased systemic oxidative stress [7] and diminished superoxide dismutase (SOD), glutathione-peroxidase (GSHpx) and glutathione (GSH) [8] are present in HFD induced obese mice. Similar results were also observed in human investigations [3]. The liver, a major site of insulin action, plays a central role in the maintenance of systemic lipid and glucose homeostasis and is especially susceptible to the damage of oxidative stress [9]. Oxidative stress-related factors may...
be implicated in the functional impairment of the liver and are associated with exacerbated nutrient oxidation [10]. An excess amount of reactive oxygen species (ROS) has detrimental effects on hepatocytes by damaging DNA, lipids, and proteins, leading to disruptions in cellular homeostasis and aggravating features of the metabolic syndrome [11, 12]. Therefore, the intake of antioxidants in order to suppress hepatic oxidative stress is needed to maintain hepatic homeostasis, which is important not only for improving insulin action in the liver, but also for eliminating insulin resistance elsewhere in the body. Apocynin (4-hydroxy-3-methoxy-acetophenone) is an efficient inhibitor of NADPH oxidase which is one major source of ROS production [13]. Its comprehensive anti-oxidative stress actions in the liver have been well-documented by numerous studies. It has protective effects on hepatic ischemia/reperfusion injury [14], hypercholesterolemia-induced hepatic oxidative burden and injury [15], hemorrhagic shock-induced liver injury [16, 17], lipopolysaccharide-induced hepatic injury, [18] and hydrophobic bile salt-induced hepatocyte shrinkage [19]. However, few studies have been conducted, thus far, to examine the antioxidant effects of apocynin in an insulin resistance model. In the present study, we investigate the effects of apocynin on oxidative stress, antioxidant enzymes, and insulin resistance in an HFD-induced insulin resistance animal model.

Materials and Methods

Animals and experimental procedures. C57BL/6J male mice were purchased from Yangzhou University (Yangzhou, Jiangsu, China) at 4 weeks of age. After a one week acclimation period, the mice were fed with either a high fat diet (60% kcal fat, 20% kcal carbohydrates, 20% kcal protein), or a normal chow diet (10% kcal fat, 70% kcal carbohydrates, 20% kcal protein, Guangzhou animal experiment center, Guangzhou, China). Mice were kept on a 12-hour light, 12-hour dark cycle and fed ad libitum. After 12 weeks of feeding, the HFD fed mice showed obvious phenotypes of insulin resistance compared to the normal diet control group (NC). We then randomly divided them into two groups (five mice per group). One group received normal water (HFD control group, HFD), while the other group received apocynin (2.4g/L) dissolved in drinking water (apocynin group, HFD-Apo) for another five weeks with HFD. Dietary intake and body weight were recorded weekly. Mice were sacrificed after a 12 hour fast. Their tissues were harvested, weighed, snap frozen in liquid nitrogen and stored at −80°C until use. All procedures were approved by the Nanjing University’s Animal Care and Use Committee.

Plasma parameters. Mice were deprived of any food overnight and blood samples were collected from the orbital sinus under anesthesia. After centrifugation at 15,000 × g for one minute, the supernatants were separated and subjected to insulin measurements using the Mouse Insulin Elisa Assay kit (Linco, Charles, MO, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as described by Hosker et al. [20], HOMA-IR = (fasting insulin (μU/mL) × fasting glucose (mmol/L)) / 22.5.

Intraperitoneal glucose tolerance tests (IPGTT). Mice were deprived of any food for six hours before IPGTT. Glucose was injected intraperitoneally at a concentration of 1g/kg body weight. Blood glucose measurements were obtained from the tail at 0, 30, 60 and 120 minutes. Blood glucose levels were determined at indicated intervals with One Touch Profile glucose meter (Johnson&Johnson, New Brunswick, NJ, USA).

Determination of lipid oxidation and antioxidant activities in plasma and liver. Malondialdehyde (MDA) levels and SOD activity in plasma and liver were measured using colorimetric kits (Nanjing Jiancheng Institute of Bio-engineering Institute, China). The activities of GSHpx, CAT and GSH content in liver were performed by colorimetric kits (Nanjing Jiancheng Institute of Bio-engineering Institute, China). Statistical analysis. Data are expressed as the means ± SEM. P values were determined by one-way ANOVA followed by LSD and differences were considered significant if p < 0.05.

Results

Characterization of the mice after apocynin treatment. After five weeks of apocynin treatment, the body weights of the HFD group and the HFD-Apo group were significantly higher than the NC group (p<0.01, respectively). No difference in body weight was observed between the HFD group and the HFD-Apo group by the first four weeks. By the fifth week, the body weight of the HFD-Apo group was markedly lower than the HFD group (p<0.05) (Figure 1A). As shown in Figure 1B, the HFD group had significantly higher serum fasting glucose (Figure 1B), fasting insulin (Figure 1C) and FFA (Figure 1D) levels compared to the NC group. The HFD-Apo group had reduced levels of the aforementioned substances when compared to the HFD group, demonstrating a significant response to apocynin administration (p<0.05, respec-
Insulin sensitivity of the mice after apocynin treatment. The area under the curve (AUC) calculated from the intraperitoneal glucose tolerance test (IPGTT) is one index of insulin sensitivity (Figure 2A, B, and C). The incremental AUC was significantly higher in the HFD group compared to the NC group (p<0.01). Apocynin treatment significantly decreased the incremental AUC in the HFD-Apo group compared with the HFD group (p<0.05). The results of HOMA-IR, another index of insulin sensitivity corresponding with fasting glucose and insulin levels, revealed significantly higher levels in the HFD group than in the NC group (p<0.01). Apocynin treatment significantly decreased HOMA-IR in the HFD-Apo group compared to the HFD group (p<0.05).

Serum oxidative stress parameters after apocynin treatment. As observed in Figure 3A, serum lipid oxidation products assessed by the MDA level were remarkably higher in the HFD group than in the NC group (p<0.05), and were reduced in the HFD-Apo group after apocynin treatment (p<0.05). Moreover, antioxidant enzyme SOD activity showed a sizable decrease in the HFD group. Apocynin treatment in the HFD-Apo group significantly upregulated SOD activity compared to the HFD group (p<0.05) (Figure 3B).

Hepatic lipid peroxidation after apocynin treatment. MDA, a marker of hepatic lipid peroxidation, was measured in the liver of C57BL/6J mice. In the
Apocynin reduced insulin resistance in high-fat diet mice

HFD group, hepatic MDA levels increased compared to the NC group. Following apocynin treatment, MDA levels significantly decreased in the HFD-Apo group compared to the HFD group (Figure 4).

**Hepatic antioxidant systems parameters after apocynin treatment.** In the HFD group, SOD activity significantly decreased compared to the NC group (p<0.05), whereas, the HFD-Apo group showed upregulated SOD activity compared to the HFD group following apocynin treatment (p<0.05) (Figure 5A). GSHpx activity was remarkably lower in the HFD group than in the NC group (p<0.05), while apocynin treatment increased the GSHpx activity in the HFD-Apo group compared to the NC group and the HFD-Apo group (Figure 5D).

**Discussions**

The current study examines the effects of apocynin on oxidative stress and antioxidant enzyme levels in HFD-induced insulin resistant mice, a classic example of the insulin resistance model [21]. We observed that 1) apocynin treatment significantly improved insulin sensitivity in HFD fed mice, 2) apocynin treatment remarkably reduced systemic oxidative stress, and 3) apocynin treatment clearly suppressed hepatic lipid peroxidation and increased antioxidant capacity.

In our study, HFD fed mice exhibited significantly higher body weight, fasting glucose and fasting insulin levels compared to those of the NC group. Following apocynin administration, we observed an improvement in fasting blood glucose and fasting insulin levels compared to those of the HFD fed control. Weight gain was more gradual in the HFD-Apo group than in the HFD group. IPGTT and HOMA-IR results directly showed that insulin sensitivity significantly improved after apocynin treatment. Furthermore, apocynin treatment significantly decreased circulating FFA when compared to the HFD group. These results indicate that apocynin improved hyperinsulinemia, hyper-
glycemia and lipid metabolism in HFD fed mice which signifies the improvement of insulin resistance. Previous studies demonstrated that apocynin treatment decreased plasma glucose, insulin and triglycerides in KKAy mice, a genetic murine model of type 2 diabetes, thus supporting our results [3]. Other studies conducted by Yokota et al. [22] and Du et al. [23] showed that although apocynin had a trend to reduce hyperglycemia in HFD fed C67BL/6 mice, there was no significant difference between the apocynin treatment group and the HFD control group. This may be explained by the different concentrations of apocynin used: they used 5mM and 10mM doses respectively, both of which are less than the 2.4g/L (nearly 14.4mM) used in our study.

Oxidative stress has been proven to be implicated in the pathogenesis of insulin resistance. Increased ROS levels are an important trigger for insulin resistance [24], which can lead to abnormal changes in intracellular signaling and contribute to the development of insulin resistance [25]. The levels of ROS depend on the balance between their rate of production and their rate of clearance by antioxidant compounds. In the present study, HFD had significantly decreased the activity of a key antioxidant SOD and increased the levels of MDA, a measure of ROS [26]. Apocynin administration remarkably upregulated the activity of SOD and reduced the level of MDA. This implies that systemic oxidative stress is suppressed by apocynin treatment which may lead to the improvement of insulin resistance.

The liver plays a central role in the regulation of whole-body glucose, fatty acid, and amino acid metabolism. However, the liver is especially susceptible to ROS damage [9]. Increased ROS can damage DNA, lipids, and proteins in hepatocytes, contributing to disruption in cellular homeostasis and aggravating metabolic syndrome features [11, 12]. The damage caused by ROS is mostly local because ROS are relatively short-lived. However, ROS can initiate lipid peroxidation within the cell, resulting in the formation of aldehyde by-products such as MDA [27]. These molecules have longer half-lives than ROS, and thus have the potential to diffuse from their site of origin to reach distant intracellular and extracellular targets, thereby amplifying the effects of oxidative stress. Moreover, ROS could then impair mitochondrial function through these reactive aldehydes, further increasing ROS and exacerbating liver damage. A ROS-dependent vicious cycle may then ensue [28]. Impaired liver function may impact the action of insulin that contributes to insulin resistance. If ROS are suppressed in the liver, then insulin sensitivity should improve [29].

In the present study, HFD fed mice showed significantly higher hepatic MDA levels, reflecting the higher ROS levels observed in previous studies [30]. Apocynin administration significantly decreased MDA levels in the liver compared to the HFD group. Apocynin has been used as an inhibitor of NADPH oxidase, a major source of ROS.
Apocynin reduced insulin resistance in high-fat diet mice

Meeting the demand to convert overload hydrogen peroxide in the liver of HFD mice. After apocynin administration, CAT activity went back to a normal level which reflects that apocynin significantly reduced hydrogen peroxide, lipid peroxidation and oxidative stress in the liver. Cederbaum et al. also found that CAT levels significantly increased after cytochrome P450 2E1 (CYP2E1) induced oxidative stress in HepG2 cell lines. They suggested that these results may reflect an adaptive mechanism to remove CYP2E1-derived oxidants which is similar to our hypothesis [41].

2) Some potential activators may stimulate the pathway to increase hepatic CAT in the HFD mice, but these mechanisms have yet to be worked out.

In conclusion, the present study demonstrates the effects of apocynin treatment on systemic and hepatic oxidative stress in HFD-induced insulin resistance in a murine model. We found that apocynin upregulates the activity of SOD and decreases the levels of MDA in the circulation. Additionally, apocynin alleviates hepatic oxidative stress through increased activity of SOD and GSHpx and decreases the activity of CAT and MDA levels in high-fat diet induced obese mice. Apocynin may attenuate systemic and hepatic oxidative stress, contributing to the improvement of hyperglycemia, hyperinsulinemia and dyslipidemia in HFD-induced insulin resistant mice.

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References


