Induction of Haemeoxygenase-1 Improves FFA-Induced Endothelial Dysfunction in Rat Aorta

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Key Words
Haemeoxygenase-1 • Free fatty acids • Endothelial dysfunction

Abstract
Background: The induction of haemeoxygenase-1 (HO-1) exerts beneficial effects in the setting of endothelial dysfunction in obesity. High free fatty acid (FFA) levels are a common feature of obesity and are the primary cause of endothelial dysfunction. The objective of our study was to explore the effects of HO-1 induction on FFA-induced endothelial dysfunction in rats.

Methods: Rats received FFA treatment with either cobalt protoporphyrin (CoPP) to induce HO-1 or stannous protoporphyrin (SnPP) to inhibit HO-1. Endothelial function was determined by measuring endothelium-dependent vasodilatation (EDV). Nitric oxide (NO) production, superoxide production and nuclear factor (NF)-κB expression in the aorta were each determined. The levels of adenosine monophosphate (AMP)-activated kinase (AMPK) and endothelial nitric oxide synthase (eNOS) expression in endothelial cells were determined via Western blotting.

Results: Induction of HO-1 by CoPP decreased circulating FFA, high-sensitivity C-reactive protein and malondialdehyde levels and increased serum adiponectin and glutathione levels compared with the FFA group (P<0.05). High FFA levels resulted in EDV impairment, which was improved by HO-1 induction (P<0.05). Induction of HO-1 increased NO levels and reduced aortic superoxide production and NF-κB expression compared with the FFA group. The FFA group exhibited decreased AMPK expression and eNOS phosphorylation, both of which were enhanced via HO-1 induction (P<0.05). The beneficial effects of CoPP on EDV were partially attenuated in vitro in the presence of inhibitors of AMPK, phosphatidylinositol 3-kinase (PI3K), and eNOS. Conclusions: HO-1 induction with CoPP improves FFA-induced endothelial dysfunction in the rat aorta. The protective mechanism appears to be related to the activation of the AMPK-PI3K-eNOS pathway as a result of increased adiponectin levels as well as decreased inflammation and oxidative stress.

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Introduction

Many clinical observations and animal experiments have demonstrated that obesity is a major worldwide health care challenge [1, 2] and is an independent risk factor for ischemic cardiovascular disease [3]. Endothelial dysfunction is considered one of the primary causes of cardiovascular disease among obese people. In the setting of obesity, the release of excessive free fatty acids (FFA) from visceral fat results in endothelial lipotoxicity via increased levels of intracellular fatty acid metabolites and decreased mitochondrial fatty acid oxidation [4]. Increased inflammation and oxidative stress also alter endothelial nitric oxide synthase (eNOS) expression and activity, resulting in reduced nitric oxide (NO) production and low NO bioavailability, both of which regulate endothelial function.

The haeme oxygenase (HO) system is the primary pathway for haeme catabolism, which drives the conversion of haeme, a potentially harmful pro-oxidant, into biliverdin, iron, and carbon monoxide [5]. There are two HO isoenzymes: HO-1 and HO-2, the latter of which is constitutively expressed and accounts for the majority of HO activity. HO-1 is inducible and is upregulated in response to both hypoxia and oxidative stress, exerting cytoprotective effects against oxidative insults [6].

Previous studies have shown that upregulation of HO-1 increases adiponectin levels by remodelling adipose tissue in both obese and diabetic rats [7, 8]. This relationship between HO-1 induction and increased adiponectin levels has resulted in the proposal of the existence of an HO-1/adiponectin axis [9-11]. Adiponectin is a cytokine secreted from adipose tissue that has both anti-atherogenic and insulin-sensitising properties [12]. Yu et al. [13] demonstrated that adiponectin improves endothelial function by increasing NO production and activating the adenosine monophosphate (AMP)-activated kinase (AMPK)-eNOS pathway. These events decrease NO inactivation by blocking superoxide production in obese rats.

HO-1 induction has also recently been found to exert beneficial effects on cardiovascular health by normalising vascular function. Cao et al. [14] demonstrated that induction of HO-1 in association with increased levels of adiponectin prevents endothelial dysfunction in obese rats by blunting oxidative stress. Induction of HO-1 also attenuates weight gain and decreases inflammatory factors in both obese rats and obese diabetic mice, exerting beneficial effects on vascular function [15]. Given that elevated FFA levels are a common feature of obesity and are a primary cause of induced endothelial dysfunction, it is somewhat surprising that we still do not know whether HO-1 exerts a direct vascular protective effect on FFA-induced endothelial function. Therefore, the objectives of this study were to explore the potential effects of HO-1 induction on FFA-induced endothelial dysfunction in rats to better understand the mechanisms underlying these processes. We tested our hypothesis using an established method that entails infusing 20% Intralipid and heparin to elevate plasma FFA levels [16] and cobalt protoporphyrin (CoPP) to induce HO-1. To verify that the effects of CoPP were due to increased HO-1 activity, we also concurrently treated the FFA group with stannous protoporphyrin (SnPP) to inhibit HO-1 activity.

Materials and Methods

Materials

Norepinephrine (NE), acetylcholine (ACh), and sodium nitroprusside (SNP) were each purchased from Sigma (St. Louis, MO, USA). Antibodies for the Western blots were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Adiponectin and high-sensitivity C-reactive protein (hs-CRP) assay kits were purchased from USCN Life Science Inc. (Wuhan, China). NO and malondialdehyde (MDA) assay kits were purchased from Beyotime Biotechnology (Beijing, China). CoPP and SnPP were purchased from Phoenix Pharmaceuticals, Inc., Burlingame, CA, US.

Experimental animals

Male Wistar rats (eight weeks-old) were purchased from Shandong Lukang Pharmaceutical Limited Company (Jining, China); their use was approved by the Institutional Animal Care and Use Committee.
(IACUC). The study conformed to the Guide for Care and Use of Laboratory Animals as published within Belgian Regulations. All of the rats were housed under standard laboratory conditions with free access to water and food. The rats were catheterised under sodium pentobarbital anaesthesia via a cannula inserted into the left carotid artery and silastic tubing inserted into the right jugular vein. All of the catheters were tunnelled under the skin and exteriorised on the upper back, and the rats were allowed to recover for 3 days before the experiments commenced. The rats were examined while awake and unstressed at 14 h following food withdrawal and then randomly divided into the following four groups: a normal control (NC) group, an FFA group, a CoPP group, and an SnPP group. The FFA group was infused for 6 h with 20% Intralipid (18 μL/min) plus heparin (0.72 IU/min) to increase plasma FFA levels (FFA treatment) [17]. The CoPP group was also subjected to FFA treatment and simultaneously infused with CoPP (800 nmol/kg/min) [18] for 6 h. The SnPP group was also subjected to FFA treatment and simultaneously infused with both CoPP (800 nmol/kg/min) and SnPP (800 nmol/kg/min) for 6 h. The NC group was infused with normal saline alone (18 μL/min). To determine the effects of CoPP or SnPP on FFA treatment, the normal rats were also infused with CoPP or CoPP and SnPP as controls, respectively. Blood samples were collected at baseline and at the end of the infusions as described below. Following the infusions, intact thoracic aortas were immediately collected.

**Evaluation of endothelial function**

As has been described previously [19-21], intact thoracic aortas were immediately dissected and immersed in Krebs-Henseleit bicarbonate buffer (K-H solution; 95% O₂ and 5% CO₂) to measure endothelial function. The thoracic aortic vascular ring was then cut into 3-mm ring segments and immersed in individual organ chambers filled with 10 mL of K-H solution at 37°C. The rings were then mounted on two steel wires, as follows: one steel wire was fixed, and the other was inserted into the lumen and attached to the chamber and to an isometric force displacement transducer (PowerLab, AD Instruments, Dunedin, New Zealand) to record changes in force. The solution was continuously gassed with 95% O₂ and 5% CO₂ and maintained at 37°C. The rings were equilibrated for 60 min with a resting tension of 1.0 g. During this time, the tissues were washed every 30 min with K-H solution. Each of the rings was first contracted with KCl (60 mM) and subsequently rinsed several times in K-H solution. Before the addition of ACh (10⁻⁴ to 10⁻² mol/L) or SNP (10⁻⁸ to 10⁻⁴ mol/L) to the solution to assess the EDV or the endothelium-independent vasodilatation (EIV) response of the arterial rings, NE (1 µM) was added to induce a steady contraction. The collected data were used to prepare concentration-relaxation response curves.

**Plasma measurements**

Plasma FFA, serum glutathione (GSH), and MDA concentrations were measured via a colorimetric assay according to the manufactures’ instructions. Serum adiponectin and hs-CRP levels were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

**Determination of total NO production**

Following the determination of endothelial function, total NO production (nitrite and nitrate) by the aortic rings was determined via a modified Griess reaction method as described previously [22, 23]. Briefly, after the ACh (10⁻⁴ M) was added, 100 μL of K-H solution was taken from the organ bath and mixed with an equal volume of modified Griess reagent. Following 10 min of incubation at room temperature, the NO concentration was spectrophotometrically determined at 540 nm according to the manufacturer’s instructions. The values were estimated per the amount of dry weight in the tissue.

**Measurement of HO activity in the aorta**

HO activity was assayed in the aortic homogenates as described previously [24]. Briefly, spectrophotometry was used to measure the concentration of bilirubin, the end product of haeme degradation, using the difference in absorption between 464 and 530 nm.

**Measurement of superoxide production in the aorta**

Superoxide production in the rat aorta was determined via the lucigenin chemiluminescence method as described previously [25, 26]. Superoxide production was expressed as counts per minute per milligram of aortic tissue.
Immunohistochemistry

Portions of the aorta were collected for NF-κB immunohistochemistry. Briefly, the aortic rings were fixed in 10% formalin, sectioned and then used for NF-κB immunohistochemistry. After restoring antigen retrieval, the sections were incubated with an anti-NF-κB antibody (Beijing Zhongshan Biotechnology Co., Ltd., Beijing, China) and a secondary antibody. Sections were then counterstained with haematoxylin and examined via light microscopy. Six fields per animal (three samples × two fields) were analysed, and the numbers of NF-κB p65-positive nuclei and cells were calculated using Image-Pro plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Western blot analysis

The extracted proteins from the aorta and the endothelial cells were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto nitrocellulose membranes and blocked with a solution of Tris-buffered saline containing 5% fat-free milk and 0.1% Tween-20 (TBST) at room temperature for 1 hour; probed with different primary antibodies against AMPK, eNOS, p-eNOS (Ser 1177), HO-1 and HO-2, and washed three times in TBST. The membranes were incubated with secondary antibodies for 1 hour, washed with TBST and stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. The protein expression levels were quantified using Image Pro-plus 6.0.

Signalling pathway studies

To investigate the potential mechanism by which CoPP affects FFA-induced endothelial dysfunction, the aortic rings obtained from the CoPP treated groups were incubated with the following specific inhibitors of the candidate pathways: (1) 20 µM of the AMPK inhibitor compound C (Merck, Darmstadt, Germany); (2) 30 µM of the PI3K inhibitor LY294002 (Sigma, St. Louis, MO, USA); and (3) 1 mM of the eNOS inhibitor L-NAME (Sigma). Following 60 min of incubation, the aortic rings were pre-contracted with norepinephrine (1 µM) followed by either ACh (10⁻⁶-10⁻⁴ M) or SNP (10⁻⁸-10⁻⁴ M) to determine the EDV and EIV.

Statistical analysis

All of the data were subjected to a one-way analysis of variance (ANOVA) statistical analysis using the SPSS 16.0 statistical package (IBM, Chicago, IL, USA), and the data were expressed as the mean ± standard deviation (SD). The Student–Newman–Keuls method was used to detect significant differences among the group comparisons. A P value of less than 0.05 was considered statistically significant.

Results

Biometric and blood parameters of rats in the study groups

We did not observe a significant difference between the FFA levels in the basal state (Table 1) (P>0.05). However, the rats in the FFA group exhibited significantly increased FFA levels following the infusion of Intralipid and heparin compared with the NC group. In contrast, the CoPP group exhibited a reduction in FFA levels compared with the FFA group (P<0.05). Additionally, the FFA group exhibited increased circulating hs-CRP and MDA levels as well as decreased serum adiponectin and GSH levels compared with the NC group (P<0.05). Interestingly, the CoPP rats exhibited decreased circulating hs-CRP and MDA levels and increased serum adiponectin and GSH levels (P<0.05). However, there were no significant differences in the above indexes between the FFA and SnPP-treated rats (P>0.05). In the normal rats treated with either CoPP or SnPP, no differences in the above indexes were observed compared with the NC group.

Endothelial function in rats

ACh-induced EDV in the thoracic aorta was attenuated in the FFA group compared with the NC group. However, the attenuation decreased following CoPP treatment (P<0.05); this effect was reversed via treatment with SnPP. Notably, no significant differences in SNP-induced EIV were noted among the four groups (Fig. 1). Additionally, in the normal rats
Table 1. Blood parameters of Rats in the Studied Groups (n=7-8). Data are shown as mean ± SD. FFAs, free fatty acids; Hs-CRP, high-sensitive C reaction protein; MDA, malondialdehyde; GSH, glutathione; NC, normal control group; FFA, the group treated with FFA; CoPP, the group treated with FFA and CoPP; SnPP, the group treated with FFA, CoPP and SnPP; NC-CoPP, normal control group treated with CoPP; NC-SnPP, normal control group treated with CoPP and SnPP; *P<0.01 vs NC group. †P<0.01 vs FFA group.

| Group          | FFA (mmol/L) (before infusion) | FFA (mmol/L) (after infusion) | Adiponectin (ug/L) | Hs-CRP (mg/L) | MDA (umol/L) | GSH (mg/L) |
|----------------|--------------------------------|--------------------------------|--------------------|--------------|--------------|-------------|
| NC             | 0.33 ± 0.11                    | 0.40 ± 0.12                    | 210.4±30.6         | 0.89±0.25    | 2.45±0.54    | 209.5±35.6  |
| FFA            | 0.34 ± 0.13                    | 1.26 ± 0.30*                   | 88.6±27.0*         | 2.05±0.59*   | 5.87±0.71*   | 156.2±30.2* |
| CoPP           | 0.36 ± 0.14                    | 0.65 ± 0.31*                   | 190.8±32.6*        | 1.08±0.45*   | 2.39±0.51*   | 220.8±40.4* |
| SnPP           | 0.40 ± 0.17                    | 1.15 ± 0.41*                   | 92.7±30.3*         | 2.19±0.48*   | 5.55±0.47*   | 163.5±28.4* |
| NC-CoPP        | 0.31 ± 0.12                    | 0.45 ± 0.10                    | 228.4±24.5         | 0.90±0.21    | 2.25±0.61    | 190.5±40.1  |
| NC-SnPP        | 0.32 ± 0.10                    | 0.43 ± 0.14                    | 218.5±21.8         | 0.95±0.27    | 2.38±0.56    | 201.7±38.9  |

Fig. 1. Vasorelaxation response of thoracic aorta segment to Ach (A) and SNP (B) in the studied groups. ACh-induced EDV in thoracic aorta was attenuated in the FFA group compared with the NC group. However, attenuation decreased following CoPP treatment and this effect was reversed by treatment with SnPP. Notably, no significant differences in SNP-induced EIV were found between the four groups. N= 7–8 vascular segments/group from 7–8 rats. Data are shown as mean ± SD. *P < 0.05 vs NC group. †P <0.05 vs FFA group.

Fig. 2. Total NO production of the endothelium from the studies groups. NO concentration was significantly reduced in the FFA group compared with the controls. However, NO levels from the CoPP group were substantially increased compared with those from the FFA group. There was no difference between the FFA and SnPP groups. N = 7–8 vascular segments/group from 7–8 rats. Data are shown as mean ± SD. *P < 0.05 vs NC group; †P <0.05 vs FFA group.

treated with either CoPP or SnPP, no differences in EDV were noted compared with the NC group (P>0.05, data not shown).
To further explore the possibility that CoPP may increase NO production, we measured the NO levels in the K-H solutions of each of the four experimental groups. As shown in Figure 2, the NO concentration was significantly decreased in the FFA group compared with the NC group ($P<0.05$). However, the NO levels in the CoPP group were significantly increased compared with the FFA group ($P<0.05$). There was no difference between the FFA and SnPP groups ($P>0.05$).

**Total NO production in the aorta**

To further explore the possibility that CoPP may increase NO production, we measured the NO levels in the K-H solutions of each of the four experimental groups. As shown in Figure 2, the NO concentration was significantly decreased in the FFA group compared with the NC group ($P<0.05$). However, the NO levels in the CoPP group were significantly increased compared with the FFA group ($P<0.05$). There was no difference between the FFA and SnPP groups ($P>0.05$).

**HO activity in the aorta**

HO activity was measured in the aortas isolated from all four groups (Fig. 3A). We observed a significant decrease in HO activity in the FFA group compared with the NC group ($P<0.05$); CoPP increased HO activity in the aorta, whereas SnPP abolished this increase ($P<0.05$).

**Superoxide production in the aorta**

Aortic superoxide production levels were increased in the FFA group compared with the NC group ($P<0.05$). In contrast, CoPP-treated rats exhibited decreased aortic superoxide...
production compared with the FFA group ($P<0.05$). Interestingly, SnPP inhibited HO activity compared with the CoPP-treated rats, which exhibited increased superoxide production (Fig. 3B).

**Immunohistochemistry**

The thoracic aortic endothelial cells exhibited increased NF-κB expression in the FFA group compared with the NC group, as determined via immunohistochemistry (positive cells, $82.6 \pm 7.4\%$ vs. $18.5 \pm 2.1\%$). However, CoPP treatment decreased NF-κB expression ($43.6 \pm 8.3\%$ vs. $82.6 \pm 7.4\%$). No significant difference was observed between the SnPP and FFA groups ($80.1 \pm 9.2\%$ vs. $82.6 \pm 7.4\%$) (Fig. 4).
Western blotting for HO-1 and HO-2 levels in the aortas
The rats in the FFA group exhibited lower HO-1 protein levels compared with the NC group ($P<0.05$, Fig. 5). CoPP treatment caused an increase in the HO-1 protein levels in the aorta, whereas SnPP treatment abolished this increase ($P<0.05$). There were no significant differences in the levels of HO-2 protein.

Western blotting for AMPK and eNOS expression in the endothelial cells
As shown in Figure 6, the FFA group exhibited decreased AMPK and eNOS phosphorylation ($P<0.05$). However, CoPP treatment enhanced both AMPK and eNOS phosphorylation ($P<0.05$).

Signalling pathway studies
As shown above, CoPP treatment improved ACh-induced EDV attenuation in the FFA group. However, the beneficial effect exerted by CoPP was partially attenuated in the presence of AMPK inhibitor compound C, the PI3K inhibitor LY294002, and the NO synthase inhibitor L-NAME (Fig. 7). SNP-induced EIV changes were not statistically significant among the four groups (data not shown).

Discussion
In this study, we demonstrated that induction of HO-1 with CoPP improves FFA-induced endothelial dysfunction in rats. The mechanism for this protective effect is related to the activation of the AMPK-PI3K-eNOS pathway, which is induced by increasing adiponectin levels in parallel with reduced inflammation and oxidative stress.

The pathogenic relationship between obesity and cardiovascular disease is well established [27]. Endothelial dysfunction, a critical component of atherosclerosis development, plays a critical role in both the onset and the development of cardiovascular disease and is observed in obesity-associated metabolic syndrome [13]. Several risk independent factors for endothelial dysfunction have been identified in the setting of obesity, including elevated circulating FFA levels [13]. Elevated FFA levels impair endothelial cell NO production by inducing chronic inflammation and oxidative stress. In our study, the rats in the FFA group exhibited significantly increased FFA levels following the infusion of Intralipid and heparin, indicating the successful establishment of the high FFA model. As expected, the ACh-induced EDV in the aorta was attenuated in the FFA group compared with the NC group, a finding consistent with previous studies [17].

The HO system exerts its antioxidant properties via its by-products: biliverdin, iron, and carbon monoxide [5]. HO-1 is upregulated in response to oxidative stress and plays a crucial protective role against oxidative insult in obesity and diabetes-induced cardiovascular disease [6, 28]. HO-1 induction prevents endothelial dysfunction by decreasing oxidant...
production [29]. Recent studies have demonstrated that HO-1 induction increases serum adiponectin levels by remodelling adipose tissue in obese and diabetic rats [7, 8, 30], a relationship referred to as the HO-1/adiponectin axis [9-11]. In our study, the HO-1 activator CoPP was used to induce HO-1 activity, whereas SnPP was used to inhibit HO-1 activity. Our results indicated that CoPP-induced HO-1 increased both EDV and serum adiponectin levels in rats infused with Intralipid and heparin. However, inhibition of HO-1 with SnPP did not significantly increase either EDV or serum adiponectin levels. These results suggest that HO-1 induction improved FFA-induced endothelial dysfunction and that this beneficial effect is associated with increased adiponectin levels.

EDV in the aorta is related to endothelial-derived NO, which is induced via eNOS phosphorylation. Previous studies have demonstrated that FFA-induced eNOS phosphorylation accounts for endothelial function in the setting of obesity [13]. We observed decreased eNOS phosphorylation and the NO concentration in the FFA group compared with the NC group. We also observed that CoPP-induced HO-1 increased eNOS phosphorylation and the NO concentration. These changes were not observed following SnPP-induced inhibition of HO-1 activity. In addition, the HO-1-induced improvement in endothelial function was abolished by the eNOS-specific inhibitor L-NAME in vitro. These results suggest that HO-1 induction protects against endothelial dysfunction by increasing NO levels via eNOS phosphorylation.

As mentioned above, HO-1 induction increases circulating adiponectin levels. Adiponectin is a cytokine secreted primarily by adipose tissue that exerts vasoprotective effects in the setting of cardiovascular disease. Some studies have shown that hypoadiponectinaemia is associated with endothelial dysfunction and that adiponectin-deficient mice exhibit impaired EDV and decreased NO production [31, 32]. Furthermore, adiponectin may independently protect against the endothelial dysfunction caused by high concentrations of FFA via the activation of the AMPK/PI3K/eNOS pathway and NO production [13, 33]. We observed that CoPP treatment enhanced both AMPK and eNOS phosphorylation and improved endothelial function, although these effects were abolished following treatment with inhibitors of AMPK, PI3K, and eNOS. These results indicate that HO-1 induction may partially improve endothelial function via the AMPK/PI3K/eNOS pathway. Additionally, FFA-induced inflammation and oxidative stress also cause endothelial dysfunction. Low adiponectin levels appear to be linked to both increased inflammation and oxidative stress [34]. In addition, induction of HO-1 by CoPP increases adiponectin and GSH levels but decreases hs-CRP and MDA levels as well as aortic superoxide anion production and NF-κB expression in rats treated with FFA, whereas SnPP-induced inhibition of HO-1 activity does not. These results suggest that HO-1 induction may improve endothelial function via increased adiponectin levels and decreased inflammation and oxidative stress. Thus, HO-1 appears to play a critical role in cellular defence against FFA-induced endothelial dysfunction.

It is interesting that we observed that induction of HO-1 with CoPP reduced FFA levels. Adiponectin reduces plasma FFA levels by enhancing the β-oxidation of FFA in muscle [35]. Therefore, inducing HO-1 may reduce circulating FFA levels by increasing adiponectin levels via the HO-1/adiponectin axis [9, 10]. In addition, our conclusion that induction of HO-1 may improve FFA-induced endothelial dysfunction is based upon studies in which we examined the acute effects of induction of HO-1 on endothelial function. This conclusion contrasts with previous studies in which transgenic mice overexpressing either HO-1 or CoPP were subjected to longer periods of HO-1 induction; these studies demonstrated that the induction of HO-1 did not improve endothelial function [36, 37]. It is possible that the acute induction of HO-1 in these vessels may have had a different effect on vascular relaxation compared with chronic induction of HO-1. A possible explanation for this finding may be related to the various actions of the by-products of HO-1 induction, including CO, on vascular function under different conditions. However, the specific molecular mechanism underlying the phenomenon mentioned above must be clarified in future studies.

In conclusion, we have demonstrated that CoPP-induced HO-1 improves FFA-induced endothelial dysfunction in rats. The mechanism underlying this protective effect is associated with the activation of the AMPK-PI3K-eNOS pathway as well as increased adiponectin.
levels and reduced inflammation and oxidative stress. In summary, these findings may have important clinical implications in the management and treatment of cardiovascular disease in obese patients.

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Disclosure Statement

The authors declare that they have no conflict of interest

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