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AUDA as a Modulator of Lipid Profile and Endothelial Function in Diet Induced Hypercholesterolemia in Rats

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Keywords

- AUDA
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- Nitric oxide synthase (NOS)

Abstract

Background: Epoxyeicosatrienoic acids (EETs) are cytochrome P450 metabolites of arachidonic acid produced by the vascular endothelium and act as important regulators of vascular tone. Objectives: to study the effect of soluble epoxide hydrolase inhibition AUDA on endothelial function in hypercholesterolemic rats. Methods: Hypercholesterolemia was induced by using Matos diet for 8 weeks and it significantly increased TC, TG, and LDL-C and decreased HDL-C. AUDA, was used with a dose of 0.35 mg/ kg for 10 weeks via gastric gavage every other day. Results: AUDA treated group has significantly lower total-C, TG, LDL-C and higher HDL-C as compared to untreated group. AUDA also improved vascular reactivity and vasodilatory response to different doses of Acetyl choline. Histopathological examination of aortic rings showed reduced development of atherosclerotic changes in the vascular wall with no effect on e-NOS expression in vascular endothelial cells. Conclusions: We can conclude that AUDA treatment has improved lipid profile and protect against development of atherosclerotic histopathological changes.

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INTREODUCTION

Hypercholesterolemia has emerged as a strong risk factor for cardiovascular disease (CVD), as it is associated with alteration of vascular structure and function through building of cholesterol within the lining of the vascular wall, leading to endothelial cell dysfunction (1).

Endothelium dysfunction (ED) has been implicated in the pathophysiology of different forms of cardiovascular diseases, including atherosclerosis, hypertension, coronary artery disease, chronic heart failure, peripheral vascular disease, diabetes, and chronic renal failure (2). ED is characterized by an imbalance of endothelium secretion of vasoactive substances toward reduced vasodilation (3). This reduced vasodilatory responses is explained by reduced production of endothelium derived relaxing factors (EDRFs) as NO, prostacyclin and reduced production of endothelium derived hyperpolarizing factors(EDHFs) as epoxyeicosatrienoic acids (EETs) (4).

EETs are cytochrome P450 metabolites of arachidonic acid produced by the vascular endothelium in responses to various stimuli such as the agonists acetylcholine (ACH) or bradykinin or by shear stress which activates phospholipase A2 to release arachidonic acid (5).

EETs are important regulators of vascular tone as they act as endothelium derived hyperpolarizing factor mainly through activation of calcium-activated potassium channels leading to potassium efflux, hyperpolarization and relaxation of vascular smooth muscle cells (VSMCs) (5).Moreover, EETs attenuate inflammatory signaling pathways in both the endothelium and vascular smooth muscle through inhibition of cell adhesion molecules (CAM) expression induced by Tumor necrosis factor-α (TNF-α) and Interleukin-1α (IL-1α). EETs are considered to be endogenous protective factors against atherosclerosis, hypertension and other vascular diseases (6) as they act as potent fibrinolytic, anti-apoptotic, pro-angiogenic, and have smooth muscle cell anti-migratory effects (7).

The main route of EETs degradation is hydration by the soluble epoxide hydrolase (sEH) to the less bioactive diols, dihydroxyeicosatrienoic acids (DHETs) (8). The development of inhibitors of sEH provides an important target for pharmacological manipulation of EETs (9). sEH inhibitors (sEHI) such as 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA) stabilize EETs, prevent their degradation and enhance their beneficial effects suggesting their potential therapeutic role in reduction of endothelial dysfunction associated with hypercholesterolemia (10).

The present study aimed to investigate the possible beneficial effects of AUDA on the plasma lipid profile and on vascular function of hypercholesterolemic rats.

1. Materials and Methods

1.1. Laboratory animals

Forty five healthy male albino rats (100±20 g), ageing from 6-7 weeks were kept in the Physiology Department Animal House, Alexandria Faculty of Medicine, Egypt. They were maintained on standard conditions (natural dark / light cycle, controlled room temperature (25±2^{o}C)). The ethical guidelines of Alexandria University on laboratory animals and the National Institutes of Health guide for the care and use of Laboratory

animals (NIH Publications No. 80-23, revised 1978) were adopted. Further, the Alexandria Faculty of Medicine Ethical Committee approval was obtained.

1.2. Induction of Hypercholesterolemia

Thirty rats were fed on high cholesterol diet (HCD) containing 1 % cholesterol (EUROMEDEX), using a slight modification of Matos diet for 8 weeks (table 1).(11, 12)

At the end of the 8th week, animals were weighed, anesthetized and blood samples were collected retro orbitally and centrifuged at 3000 rpm for 10 min to separate plasma and serum. Levels of TGs (13), total cholesterol (Total-C) (14)and high-density lipoproteins (HDL) (15) were measured by colorimetric method**.** While, low-density lipoproteins (LDL) were calculated using Friedewald equation (16). Hypercholesterolemia was statistically proven.

1.3. Study design:

A group of 15 rats were fed on standard rat chow throughout the study and served as controls. While Hypercholesterolemic (HC) rats were randomly divided into two groups (15 rats each): AUDA-treated HC rats; this group was given AUDA (Sigma-Aldrich) (0.35 mg/ kg), by oral gavage every other day for 10 weeks (6). Untreated HC group received distilled water through gavage every other day for 10 weeks.

1.4. Animals sacrifice and sampling:

At the end of the 18th week, animals were weighed and anesthetized with ketamine (80 mg/kg) plus xylazine (10 mg/kg I.P.) (17). Then rats were dissected removing the internal organs located in the thoracic and abdominal regions carefully to avoid damages to the aorta, which is closely located with the spinal cord. Adipose and connective tissues around the aorta were removed. The aorta was gently excised and placed in a petri dish containing Kreb's solution of pH 7.4.Then it was cut into aortic rings of approximately 3–5 mm width.

Blood samples were collected, centrifuged at 3000 rpm for 10 min to separate plasma and serum. Levels of TGs (13), total cholesterol (Total-C) (14)and high-density lipoproteins (HDL) (15) were measured by colorimetric method**.** While, low-density lipoproteins (LDL) were calculated using Friedewald equation (16).

1.5. In vitro testing of the aorta (Isometric tension study):

The endothelium function was assessed by testing the relaxation produced by Ach in rings pre-contracted with norepinephrine. Following equilibration, cumulative concentrations (10^{-6} to 10^{-3} mol/l) of NE were added to obtain a dose-response curve (constrictor response). Then, cumulative concentration response curves for the relaxant effect of Ach (10^{-9} to 10^{-3} mol/l) on the NE pre-contracted rings were recorded. Responses were expressed as a percentage of relaxation by reduction of NE-peak response. Data were acquired by a Power Lab 8/35 data acquisition system (Model No PL3508/P, AD Instruments Pty Ltd, Castle Hill, Australia). (18-21)

Table (1): Component of high-cholesterol diet (HCD) (g/1000g of diet):

Component	(Gram %)
Casein	120
Corn starch	429.6
Corn oil	250
Cholesterol	10
Salt mixture	40
Vitamin mixture	10
Cellulose	130
Total calories	4538.4

1.6. Histopathological assessment of aortae from the test rats:

1.6.1. Histological grade for atherosclerotic lesions;

Transverse sections were obtained from the abdominal aortae isolated from the studied groups, then 5 µm-thick sections were cut and stained with the conventional haematoxylin and eosin (H&E) stain. Sections were then examined using light microscopy for histopathological assessment. The histologic changes were graded into 6 types according to the American Heart Association classification of human atherosclerotic lesions (Stary et al. 1995)(22):

Type I (initial) lesion: isolated macrophages, foam cells.

Type II (fatty streak) lesion: mainly intracellular lipid accumulation.

Type III (intermediate) lesion: type II changes and small extracellular lipid pools.

Type IV (atheroma lesion): type II changes and a core of extracellular lipid.

Type V (fibroatheroma) lesion: lipid core and fibrotic layer, or multiple lipid cores and fibrotic layers, or mainly calcific, or mainly fibrotic.

Type VI (complicated) lesion: surface defects, hematoma, hemorrhage, and thrombus.

1.6.2. Immunohistochemistry for e-Nos

The deparaffinized aortic tissue sections were rehydrated in graded alcohols. Immunohistochemical staining was performed using an avidin–biotinylated immunoperoxidase method and according to the manufacture protocol. The endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min. For antigen retrieval, sections were microwaved in 10 mmol/l citrate buffer (pH 6.0). The primary antibody, e-Nos (rabbit monoclonal antibody) was then applied in a concentration of 1/100. The bound antibodies were detected using UltraVision Detection System Anti-Polyvalent, HRP/DAB. e-Nos expression was evaluated semi quantitatively in endothelial lining and expressed as Percentage of the cells with positive e-NOS expression.

1.1. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analyses were performed with IBM SPSS statistics, version 21.0 (IBM Inc.). The results were analyzed by one-way analysis of variance (ANOVA) followed by a LSD post-test for multiple comparisons and p-value ≤ 0.05 was defined to be statistically significant.

1. Results

1.1. Serum lipid profile after 8 weeks (post diet)

In this study, the effect of diet was assessed after 8 weeks to prove hypercholesterolemia according to lipid profile changes before and after diet. Our results showed significant increase in Total-C with mean value of 111.48±21.01 mg/dl as compared to the control group 87.26±16.58 mg/dl (P=0.0013). Also, there was a marked increase in TG with mean value of 102.22±45.47 mg/dl as compared to control with a mean 80.70±13.61 mg/dl (P=0.023). In addition there was a marked reduction of the protective HDL-C in high cholesterol fed rats versus the control group (P=0.049). Moreover, the increase in LDL-C and non HDL-C was highly significant in comparison with the control group (p=0.000007 and P=0.000001), respectively (figure 1).

1.2. Serum lipid profile after 10 weeks of treatment:

Ten weeks oral treatment with AUDA, produced a significant decrease in Total-C as compared to untreated HC rats (P<0.001).However, Total-C was still significantly higher than control rats (P < 0.05). TG level also was significantly elevated in untreated HC rats (P<0.05) while in AUDA treated rats there was no statistically significant difference versus control group (P=0.495). Meanwhile, there was highly significant difference between AUDA treated and untreated groups (P<0.001).

HDL was significantly higher in AUDA treated group than control group (P < 0.001) and untreated HC group (P < 0.05). AUDA treated group showed a significant decrease of LDL in comparison to the untreated group (P < 0.05). However, there was no significant difference between AUDA treated group and control group (P = 0.625). As regards non HDL-C, AUDA treated group showed significant decrease in non HDL-C in relation to untreated HC group (P < 0.05).There was no significant difference between control group and AUDA treated group (P=0.169) but the difference between control group and the untreated group was highly significant (P < 0.001) (figure 2).

1.3. Changes in aortic rings vascular reactivity in terms of contraction responses to cumulative doses of NE:

Cumulative concentrations of NE (0.25×10^{-3}-1×10^{-3} mol/l) induced vasoconstriction in a dose-dependent manner in aortae isolated from the normal control group, with maximum contraction achieved at the dose of 1×10^{-3} mol/l in the three studied groups. There was no significant difference between the three studied groups (P = 0.328, P = 0.203, P = 0.894) for the three doses of NE, respectively (Figure 3A).

1.4. Percentage relaxation of NE pre-contracted rings in response to cumulative doses of Ach:

The maximally contracted rings showed a dose-dependent relaxant response to cumulative doses of Ach (10^{-9} to 10^{-3} mol/l). The untreated HC group showed a significant amelioration of vascular reactivity in comparison to the control group and AUDA treated group (P<0.001). There an enhanced ACh-induced relaxant responses in the AUDA treated group for the three doses of Ach. There was no difference between the control group and the AUDA treated group in the three doses of Ach (P = 0.632, P = 0.291, P = 0.847) (Figure 3B).

1.5. Histopathological assessment of aortae from the test rats:

1.5.1. Histological grade for atherosclerotic lesions;

The examined control group showed normal endothelial lining with unremarkable vessel wall. While the hypercholesterolemic untreated group showed evidence of fat deposition in the vessel wall ranging from isolated macrophages infiltration in the wall (stage I) in (6.6 %) of cases, fatty streaks (stage II) in (46.6 %) of cases. Stage III was evident in 33.3 % of cases and stage IV in 13.3% of rats. Whereas, the AUDA treated group showed an evident decreased fat deposition, stage I was detected in (26.6 %) of cases, fatty streaks (stage II) in (60 %) of cases, stage III in only 6.6% of rats with no evident stage IV in any of the treated rats (Figure 4 A, C, E).

3.5.2. Immunohistochemistry for e-NOS;

Positive staining for e-Nos was noted in the membrane of endothelial cells. The examined control group showed a positive staining for e-Nos with a mean value of 95% of cells with positive e-NOS expression. While e-Nos expression was significant decreased in the endothelial lining of the aortic rings of the AUDA treated and untreated HC rats as compared to the control group (P<0.001). Moreover, there was no statistical significant difference between AUDA treated and untreated HC rats (P< 0.099) implying that AUDA has no effect on e-NOS expression (Figure 4 B, D, F, G).

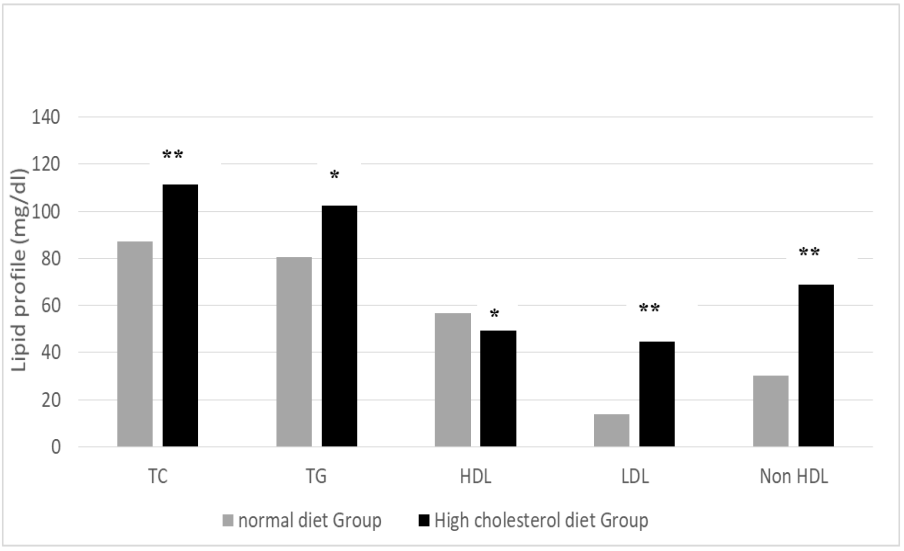

Figure (1); Effects of 8 weeks Matos diet on lipid profile in diet-induced hypercholesterolemic rats versus normal diet group. **: highly significant P ≤0.01 *: Statistically significant at P <0.05.

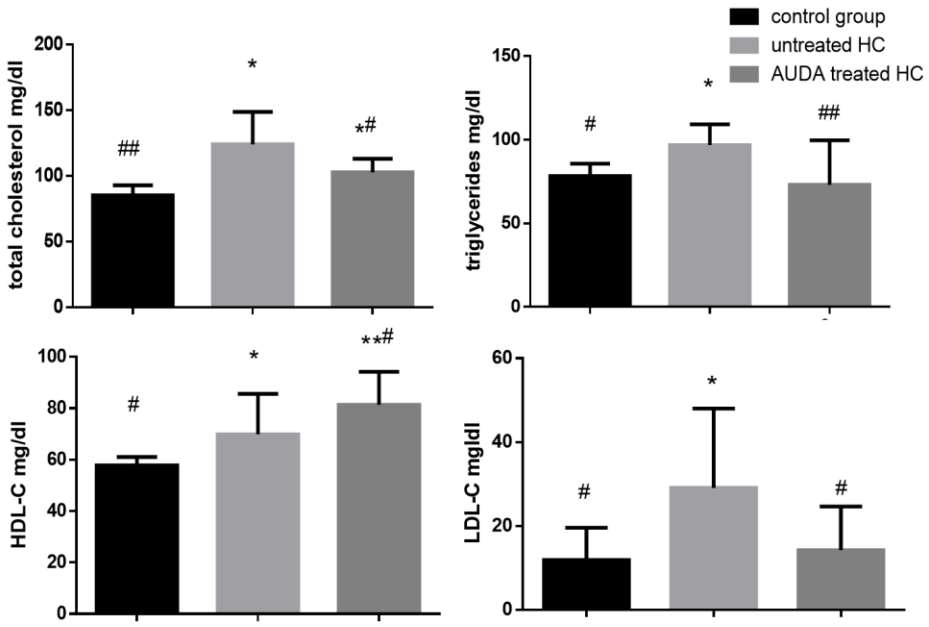

Figure (2); Effects of 10 weeks AUDA treatment on lipid profile in diet-induced hypercholesterolemic rats. Data are expressed as Mean ± SD and analyzed by ANOVA test; *: Statistically significant at P<0.05 versus control group, ** statistically highly significant at P<0.001 versus control group, # statistically significant at P<0.05 versus untreated HC group, # # statistically highly significant at P<0.001 versus untreated HC group.TC: total cholesterol; TG: Triglycerides; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol.

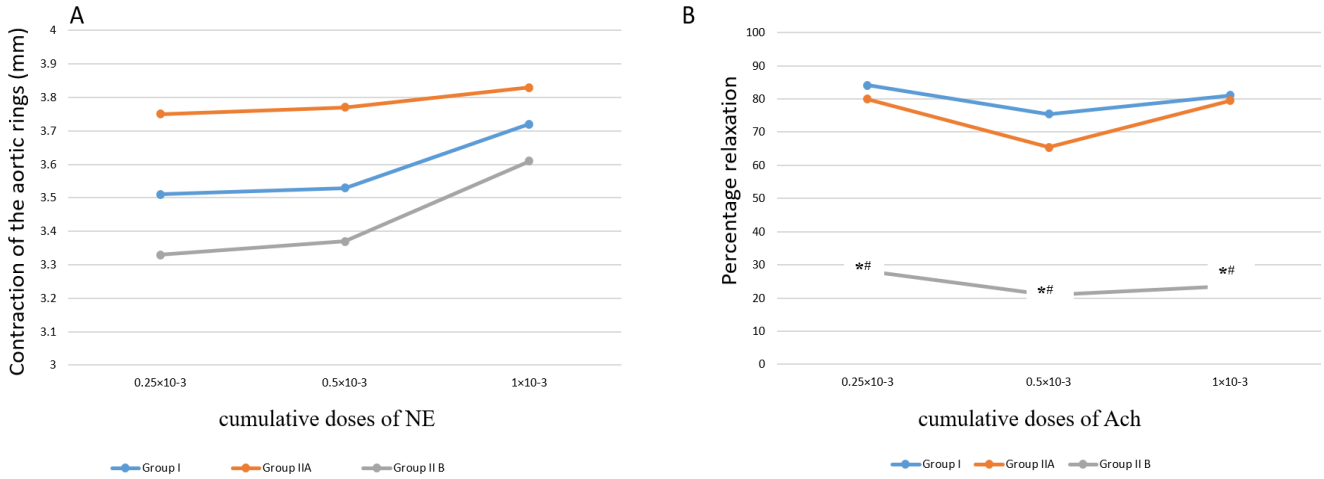

Figure (3);vascular response to different doses of NE and Ach (A) Aortic rings contraction in response to cumulative doses of NE in the different studied group. Figure (2B): percentage relaxation of NE pre-contracted rings in response to cumulative doses of Ach. *P< 0.05 versus control group, # P < 0.05 versus AUDA treated group. NE : Norepinephrin ; Ach: Acetylcholine.

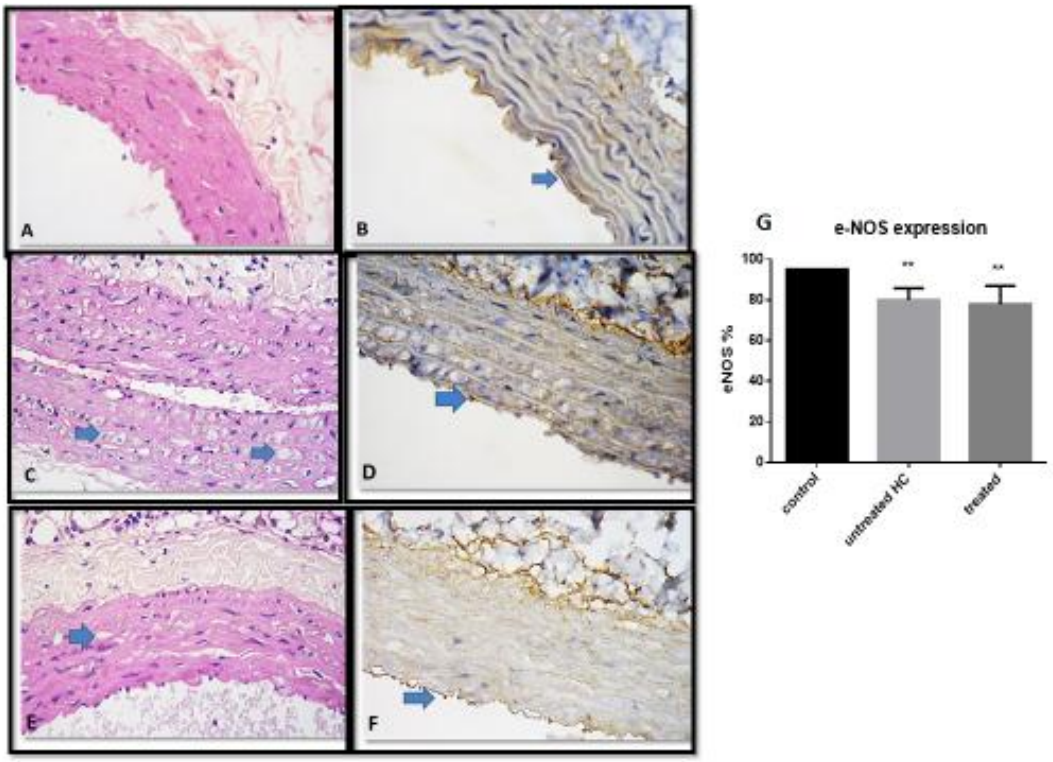

Figure (4); Histopathological assessment of rats' aortae. (A) Control group showing normal endothelial (H&Ex400). (B) Positive e-Nos staining in endothelial cells of the control group (arrow). (C) Hypercholesteolemic group showing extracellular lipid accumulation (stage IV) (H&Ex400). (D) Decreased e-NOS staining in endothelial cells of the HC untreated group (arrow). (E) Treated group showing decreased lipid accumulation (stage II) (H&Ex400). (F) Decreased e-NOS staining in endothelial cells of AUDA treated group (arrow).(G): Graphical presentation of immunohistochemistry for e-NOS expression.

2. Discussion

In the current work, HC was successfully induced by modified Matos diet fed to rats for 8 weeks and it significantly increased their Total-C, TG, and LDL-C and decreased HDL-C as compared to control group fed normal rat chow.

After 10 weeks of AUDA treatment to the HC rats, rats had a significantly lower level of Total-C, TG, LDL-C and higher HDL-C level. This

modulation of plasma lipid profile in hypercholesterolemic rats could contribute to the attenuation of atherosclerosis. The mechanisms involved in AUDA modulation of lipid profile has been intensely studied, It could be attributed to the fact that the sEH inhibition cause activation of protein kinase (AMPK) which subsequently cause inhibition of Sterol regulatory element binding proteins (SREBPs). SREBP regulate the expression of all three lipid metabolizing factors, HMG CoA reductase, fatty acid synthase and low density lipoprotein receptor (23).

The atheroprotective effect of AUDA has been shown through its effect on HDL-C. HDL plays a key role in reverse cholesterol transport (RCT) by promoting cholesterol efflux from peripheral cells, and delivering the cholesterol to the liver for excretion (24). This effect was previously explained by Shen L et al (25), who showed that sEHI increases EETs which upregulate ATP-binding cassette transporter A1 (ABCA1). ABCA1 is important for the initial step of HDL biogenesis by facilitating the efflux of cellular free cholesterol to apolipoprotein A-I (apoA-I), forming nascent HDL particles.(26) Thus, ABCA1 causes an elevation of circulating HDL-C which promote removal of excess cholesterol from the periphery into the RCT pathway for excretion and thus decreasing LDL-C and improving HDL-C/ LDL-C ratio. In accordance with our study, Zhang J et al(6) showed also a significant reduction in TG after 0.35mg/kg AUDA treatment for 10 weeks, However, they use a different animal model. They used ApoE-/- mice not diet induced hypercholesterolemia as in the present work.

Hypercholesterolemia is associated with vascular inflammation and initiation of atherosclerosis which causes endothelial dysfunction. In the current study, AUDA does not only modulate lipid profile but also improve endothelial function as assessed by the percentage relaxation of aortae pre-constricted with norepinephrine to different doses of acetylcholine via power lab AD Instrument. There was significant difference between the control group and the AUDA treated HC group versus the untreated HC group regarding the three doses 10^{-3}, 10^{-6}, 10^{-9}M of Ach. Previous studies reported a protective effect of sEH inhibitors on the endothelial function in different animal models. Iyer A et al. and Zhang LN et al. found a protective effect of sEH inhibiton on endothelial function of diabetic rats and rats with metabolic syndrome by increasing EETs/DHETs ratio. (27, 28). Roche C et al. reported that inhibition of sEH reverses coronary endothelial dysfunction and prevents the early development of cardiac hypertrophy and diastolic dysfunction in a murine model of insulin resistance by increasing EET availability but without complete restoration of BK_{Ca} channels activity (29).

Yang L et al. explained that sEH inhibition can augment bradykinin-induced vasodilation in human vessels both in vitro and in vivo, suggesting that sEH inhibition may be a novel therapeutic target to ameliorate cardiovascular risk in patients with COPD and smoking-related endothelial dysfunction by increasing 11,12-EETs the most potent regioisomer (30).

We further investigated the effect of AUDA treatment on vascular wall histopathological changes regarding the presence of foam cells, lipid accumulation and fibrotic layer. The atherosclerotic grading showed a statistically significant atherosclerotic changes seen in the

H&E staining of the aotic rings of the AUDA treated and untreated HC rats as compared to the control group. However, AUDA treated rats showed significantly reduced histopathological changes as compared to untreated HC rats indicating the efficacy of AUDA treatment in reducing atherosclerotic development.

The improved endothelial function could be attributed to increased EETs rather than increased expression of e-NOS as assessed by immunohistochemical staining. eNOS is responsible of production of NO in endothelial cells (31) . NO is a potent vasodilator in the vasculature, and the balance between nitric oxide and various endothelium derived vasoconstrictors and the sympathetic nervous system maintains blood vessel tone. Optimal levels of NO are needed for healthy endothelial cells and vessel wall (32).

Normally there is a balance between the vasodilators EETs and NO to modulate vascular tone (33). The increased EETs exhibits decreased endothelial NO synthesis to protect vascular tone (34). Thus, in our study AUDA treatment as a sEH inhibitor causes an increase in EETs which improve the vasodilatory response without increasing e-NOS expression. This goes together with data from the study of Huang Yet al (34) which showed that Inhibition of sEH by TUBS protected the impaired myogenic response in cirrhotic rats by decreasing endothelial NO synthesis, Also Qin Jet al. and Froogh G et al. showed that increased levels of EETs can decrease endothelial NO synthesis in mesenteric or coronary arteries in sEH-Knock out mice (35) (36).

This effect is not only limited to eNOS but Hung TH et al. also found that AUDA treatment reduced iNOS expression in primary microglial cultures in response to LPS or interferon gamma (IFN-γ) stimulation. Also, deletion of sEH decreased the release of NO in IFN-γ–stimulated primary microglial cultures (37).

3. Conclusions:

We can conclude that AUDA treatment causes protection against EET degradation potentiating its beneficial functions regarding improving lipid profile and protect against development of atherosclerotic histopathological changes. AUDA also has improved endothelial function, vasodilatory response and vascular reactivity.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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