Biochemical and Biophysical Research Communications 458 (2015) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Endoplasmic reticulum stress involved in high-fat diet and palmitic acid-induced vascular damages and fenofibrate intervention

Yunxia Lu [a, b, *, 1], Jingjing Cheng [a, 1], Li Chen [a, d], Chaofei Li [a], Guanjun Chen [a], Li Gui [b], Bing Shen [c], Qiu Zhang [e]

[a] Department of Biochemistry and Molecular Biology, Anhui Medical University, Hefei, Anhui 230032, PR China
[b] The Comprehensive Laboratory, Anhui Medical University, Hefei, Anhui 230032, PR China
[c] Department of Physiology, Anhui Medical University, Hefei, Anhui 230032, PR China
[d] Department of Medical Laboratory, Anhui Provincial Hospital, Hefei, Anhui 230001, PR China
[e] Department of Endocrinology, First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230022, PR China

ARTICLE INFO

Article history:
Received 16 December 2014
Available online 12 January 2015

Keywords:
Endoplasmic reticulum stress
Endothelium-dependent vasodilatation
Fenofibrate
Endothelial nitric oxide synthase
High-fat diet
Palmitic acid

ABSTRACT

Fenofibrate (FF) is widely used to lower blood lipids in clinical practice, but whether its protective effect on endothelium-dependent vasodilatation (EDV) in thoracic aorta is related with endoplasmic reticulum (ER) stress remains unknown. In this study, female Sprauge Dawley rats were divided into standard chow diets (SCD), high-fat diets (HFD) and HFD plus FF treatment group (HFD + FF) randomly. The rats of latter two groups were given HFD feeding for 5 months, then HFD + FF rats were treated with FF (30 mg/kg, once daily) via gavage for another 2 months. The pathological and tensional changes, protein expression of eNOS, and ER stress related genes in thoracic aorta were measured. Then impacts of palmitic acid (PA) and FF on EDV of thoracic aorta from normal female SD rats were observed. Ultimately the expression of ER stress related genes were assessed in primary mouse aortic endothelial cells (MAEC) treated by fenofibric acid (FA) and PA. We found that FF treatment improved serum lipid levels and pathological changes in thoracic aorta, accompanied with decreased ER stress and increased phosphorylation of eNOS. FF pretreatment also improved EDV impaired by different concentrations of PA treatment. The dose- and time-dependent inhibition of cell proliferation by PA were inverted by FA pretreatment. Phosphorylation of eNOS and expression of ER stress related genes were all inverted by FA pretreatment in PA-treated MAEC. Our findings show that fenofibrate recovers damaged EDV by chronic HFD feeding and acute stimulation of PA, this effect is related with decreased ER stress and increased phosphorylation of eNOS.

1. Introduction

Macrovascular disease is one of the common determinants of morbidity and mortality in obesity-induced metabolic syndrome [1]. However, the exact mechanisms in the development of macrovascular diseases have not been completely elucidated until now. Endothelial dysfunction is an early biomarker of vascular injury, usually develops by the loss of normal endothelium-dependent vasodilatation (EDV) responses to acetylcholine (Ach). Elevated free fatty acid (FFA)-reduced endothelial NO bioavailability is the leading factor in obesity-induced endothelial dysfunction suggested by some studies [2,3].

Endothelial cells constitute the innermost layer of blood vessels, so damage to endothelial cells is usually considered as the initial step of endothelial dysfunction. Endothelial cells with a well-developed endoplasmic reticulum (ER) have a powerful ability for protein synthesis. Recent studies reported that FFAs could result in ER stress and endothelial dysfunction [4,5], but whether FFA-induced ER stress participate in EDV has yet no reports. One of aims of the present study was to determine whether FFA-mediated ER stress could affect EDV responses in aortas of obese rats.

Abbreviations: FF, fenofibrate; EDV, endothelium-dependent vasodilatation; ER, endoplasmic reticulum; SCD, standard chow diets; HFD, high-fat diets; HFD + FF, HFD plus FF treatment group; PA, palmitic acid; FA, fenofibric acid; eNOS, endothelial nitrc oxide synthase.

* Corresponding author. Department of Biochemistry and Molecular Biology, The Comprehensive Laboratory, Anhui Medical University, No.81, Meishan Road, Hefei, Anhui 230032, PR China. Fax: +86 551 65168340.
E-mail address: wwwdluyx@sina.com (Y. Lu).
[1] These authors (Yunxia Lu and Jingjing Cheng) contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.12.123
0006-291X/

Fenofibrate (FF), a third generation of fibric acid derivatives and a specific PPARα agonist, can be used to treat diabetic vascular complications. Subgroup analyses in both the FIELD and ACCORD lipid trials suggest that FF is one of the best drugs to treat patients with atherogenic dyslipidemia [6], but the protective effects of FF on EDV and concrete mechanisms is still not known. We hypothesize that FF treatment can obviously improve EDV responses to Ach and endothelial dysfunction via alleviating ER stress in endothelial cells induced by high-fat diet (HFD) and high circulated FFAs.

HFD usually induces obesity and hypertriglyceridemia more easily in male SD rats than in female SD rats, because females experienced slower adipose gains compared with males when fed obesogenic diets [7]. We adopted an international standard HFD (D12451) to feed female SD rats for consecutive 5 months to make them obese, then they were treated with FF for another 2 months. Saturated fatty acid palmitate (16:0) is the main saturated fatty acid in the HFD, while fenofibric acid is the main metabolic form of FF *in vivo* [8], so they were chosen to treat primary mouse aortic endothelial cells (MAEC) to probe the molecular mechanisms of protective effects of FF on thoracic aorta. As far as we know, this is the first study in literature which discusses all these parameters in one single study protocol.

2. Materials and methods

2.1. Experimental animals and protocols

All animal procedures were performed in accordance with the principles approved by the Animal Ethics Committee of Anhui Medical University. Eight-week-old female Sprague Dawley rats (specific pathogen-free) were purchased from the Experimental Animal Center of Anhui Medical University (Anhui, China) and acclimated for 1 week. The rats were housed in plastic cages (5 rats/cage) at room temperature (22–25 °C) and 35–60% humidity under a 12:12 h light and dark cycle. All of them were allowed free access to laboratory chow and water. The rats were randomly divided into three groups including standard chow diet (SCD, D12450B), high-fat diet (HFD, D12451) and fenofibrate treatment group (HFD + FF, F6020, Sigma). The rats in HFD and HFD + FF group were given a standard high-fat diet (D12451) *ad libitum* for 5 months, then HFD + FF group underwent intragastric administration with FF (30 mg/kg/d) for another 2 months. Body weight and food intake were measured once a week throughout the investigation.

2.2. Serum biochemical parameters and nitrate measurements

Intact thoracic aortas were immediately collected for vascular tension measurement after all rats were fasted and anesthetized, the rest were used to determine pathological changes or gene expression. Serum samples were collected for biochemical analyses, including triglyceride (TG), total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C) and low density lipoprotein-cholesterol (LDL-C). Serum NO levels were measured according to the commercial kit instructions (Nanjing Jiancheng Bioengineering Institute, China). Free fatty acid (FFA) and palmitic acid (PA) levels were measured by gas chromatography according to the method of Han et al. [9].

2.3. Histological and lipid deposition examinations

Thoracic aorta samples were fixed in 10% formalin, embedded in paraffin, sectioned (thickness of 5 μm), stained with hematoxylin and eosin (H&E, collagenous fiber), Oil-red O staining (neutral lipid) [10] and Weigert staining (elastic fibers and integrity) [11] respectively. Sections were photographed and positively stained areas of Oil-red O was quantified by Image Pro Plus software.

2.4. Preparation of aortic rings and vascular tension experiment

Isometric tension experiment was performed as described by Dou et al. [12]. Arteries were stimulated with phenylephrine (PE, 10^{-6} mol/L) followed by cumulative concentrations of acetylcholine (10^{-8}–10^{-3} mol/L) to assess endothelial function. The recorded relaxation curves were plotted as percentages of the contraction values induced by PE. A modified cotton-tipped applicator was used to denude of endothelium (-endothelium). In order to test the role of eNOS in impaired endothelium-dependent relaxation in rat vessels, aorta from normal SD rats were incubated with *L*-NAME (100 μmol/L, N5751) for 30 min and then EDV was assessed.

In order to confirm that whether elevated PA was responsible for arterial dysfunction and FF had the protective effects, thoracic aorta were obtained from 10-week old female SD rats to assess the effects of the saturated fatty acid PA (P5585, Sigma) and FF on normal arterial function. Rats were anesthetized and vessels were obtained and mounted as described above. After FF (0.1 mM) or ER stress inhibitor 4-PBA (10 mM) were pretreated for 45 min or for 20 min respectively, the rings of thoracic aorta were kept in different concentrations of PA (0.05–0.2 mM) or ER stress inducer tunicamycin (TM, 1 μg/ml, 93755, Sigma) solution for 90 min, then EDV was assessed.

2.5. Cell culture, MTT and NO colorimetric assay

The primary mouse aortic endothelial cells (MAEC) were obtained and cultured as before [4]. MTT colorimetric assay was used to determine the dose and time-dependent effect of PA and fenofibric acid (FA, CDS003219, Sigma) on cell proliferation. PA was coupled to fatty acid-free BSA in the ratio of 2:1(PA: albumin) before experiment, equal concentrations of PA-free albumin was used as vehicle controls. The cells were firstly treated with 0, 0.1, 0.2, 0.4, 0.8 and 1.0 mM PA then 0.5 mM PA was used to observe time-dependent (6, 14, 18, 20, 24h) of PA and concentration-dependent of FA (10–200 μM) protective effect respectively. After concentration of PA and FA were determined, NO level in supernatant was assayed according to the kit instruction.

2.6. RT-PCR

Thoracic aorta and MAEC were used to analyse the mRNA expression by RT-PCR (GRP78 and CHOP) respectively. Then the genes engaged in ER stress such as IRE1α (*IRE1α*), downstream effectors such asand XBP1 (*Xbp1*, included *unspliced Xbp1*, *Xbp1u and spliced Xbp1*, *Xbp1s*) were measured in MAEC. Primer sequences were shown in Table 1. GAPDH was used as internal reference. PCR products were analyzed on a 3% agarose gel. TM (1 μg/ml) and 4-PBA (10 mM) was used as inducer and inhibitor of ER stress in cell lines respectively.

2.7. Western blot

The thoracic aorta and MAEC were used to analyze the protein expression of eNOS and phosphorylation of eNOS (p-eNOS), ERK and phosphorylation of ERK (p-ERK), JNK and phosphorylation of JNK (p-JNK), GRP78, CHOP respectively. GAPDH was used as internal reference. Antibody binding was detected by enhanced chemiluminescence (ECL) and quantified with Gel-Pro Analyzer 3.1 software (Informax). Each experiment was repeated for three times.

Table 1
Primer sequences used for RT-PCR analysis in MAEC and rat thoracic aorta.

Species and gene	Reference sequence	Primers (5′–3′)	PCR product length (bp)
Mouse CHOP	NM_007837.3	Forward: ATATCTCATCCCCAGGAAACG Reverse: TCTTCCTTGCTCTTCCTCCTC	188
Mouse GRP78	NM_022310.3	Forward: TGTGGTACCCACCAAGAAGTC Reverse: TTCAGCTGTCACTCGGAGAAT	220
Mouse IRE1α	NM_023913.2	Forward: CTGTGGTCAAGATGGACTGG Reverse: GAAGCGGGAAGTGAAGTAGC	208
Mouse XBP1	NM_013842.3	Forward: ACACGCTTGGGAATGGACAC Reverse: CCATGGGAAGATGTTCT GGG	172
Mouse GAPDH	NM_008084.2	Forward: TGTGTCCGTCGTGGATCTGA Reverse: CCTGCTTCACCACCTTCTTGAT	77
Rat CHOP	NM_001109986.1	Forward: TGGAAGCCTGGTATGAGGATCTG Reverse: GAGGTGCTTGTGACCTCTGCTG	175
Rat GRP78	NM_013083.2	Forward: TCAGCCCACCGTAACAATCAAG Reverse:TCCAGTCAGATCAAATGTACCCAGA	88
Rat GAPDH	AC_000073.1	Forward: TGCCACTCAGAAGACTGTGG Reverse: TGTGAGGGAGATGCTCAGTG	575

2.8. Statistical analysis

All data were expressed as means ± S.D. Difference between two groups or more groups were assessed using unpaired, two-tailed student's t-test or ANOVA. It was considered statistically significant when $P < 0.05$.

3. Results

3.1. Fenofibrate reduces obesity, and improves blood characteristics of HFD-fed rats

All female rats started the experiment with similar body masses. Actual food intake showed no overt differences between the three groups ($p > 0.05$), but 7 months of HFD feeding caused significantly more body mass, higher serum levels of TG, TC, LDL-C, FFA, PA, NO and lower level of HDL-C, while 2 months of FF treatment (30 mg/kg) clearly reduced body mass and reversed serum indices, but the level of TC between the 2 groups had no statistically difference ($p > 0.05$) (Fig. 1A, Table 2). Because the HFD-fed female rats showed increased plasma FFA levels, especially PA levels, we selected PA as a representative HFD ingredient to study its effects in mouse aortic endothelial cells (MAEC).

3.2. Fenofibrate improved pathology and lipid deposition in thoracic aorta

Further examination showed pathological changes including irregular thickening of thoracic aortic wall, disorders and fractures of elastic fibers in the HFD-fed female rats, in the same time these pathological changes were improved in the HFD + FF female rats (Fig. 1B). Oil red-O staining and quantification confirmed that more lipid deposited in the vascular walls of HFD group (Fig. 1B, C), while the lipid droplets were smaller in HFD + FF group. This result was consistent with serum lipid indices in Table 2.

Table 2
Effects of fenofibrate on serum parameters of female SD rats.

Parameter	SCD	HFD	HFD+FF
Initial body weight (g)	289.2 ± 14.7	355.2 ± 19.8	355.9 ± 20.3
Final body weight (g)	325.1 ± 20.3	426.6 ± 26.3**	360.7 ± 21.8$^{\#}$
TG (mmol/L)	0.24 ± 0.06	0.55 ± 0.05**	0.25 ± 0.06$^{\#\#}$
TC (mmol/L)	1.44 ± 0.09	2.48 ± 0.22**	2.06 ± 0.20
HDL-C (mmol/L)	0.94 ± 0.19	0.56 ± 0.09*	1.1 ± 0.17$^{\#\#}$
LDL-C (mmol/L)	0.21 ± 0.04	0.46 ± 0.09*	0.28 ± 0.05*
Free fatty acid (mmol/L)	0.34 ± 0.05	3.38 ± 0.14*	1.67 ± 0.08$^{\#\#}$
Palmitic acid (μg/ml)	201.75 ± 18.34	654.73 ± 40.19*	351.30 ± 31.65$^{\#\#}$
NO (μmol/L)	22.81 ± 2.18	111.88 ± 19.29**	25.62 ± 1.21$^{\#\#}$

All values shown in table are means±S.D. n=6 animals in each group.$^{*}p<0.05$: *vs* SCD group;
$^{**}p<0.01$: *vs* SCD group; $^{\#}p<0.05$: *vs* HFD group; $^{\#\#}p<0.01$: *vs* HFD group.

3.3. Effect of fenofibrate on HFD-mediated impaired vasodilation

Next we designed vascular tension experiment to test the vasodilatation activity of FF in PE constricted aortic rings from the three groups. The cumulative addition of Ach (10^{-8} to 10^{-3} M) on PE-constricted aorta caused concentration-dependent relaxation. EDV reaction to Ach were much weaker in aortic rings from the

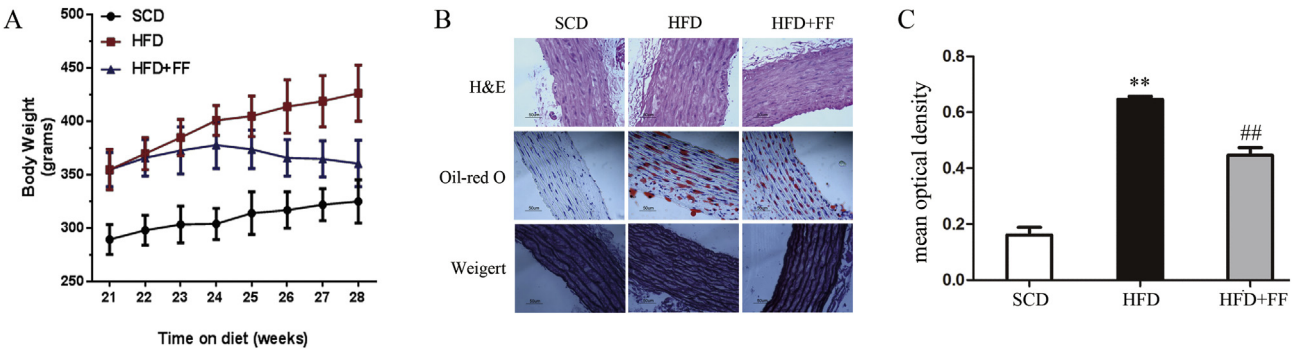

Fig. 1. Effects of fenofibrate on body mass and thoracic aorta of HFD-fed female SD rats. A. Effects of fenofibrate on body mass of HFD-fed female SD rats. B. Pathological changes in the thoracic aorta were evaluated with H&E, Weigert and Oil-red O staining (×400) respectively. Representative images were shown. C. Quantification of Oil-red O staining. Data were expressed as means ± S.D. of six female rats/group. $^{**}p < 0.01$ vs. SCD group, $^{\#\#}p < 0.01$ vs. HFD group.

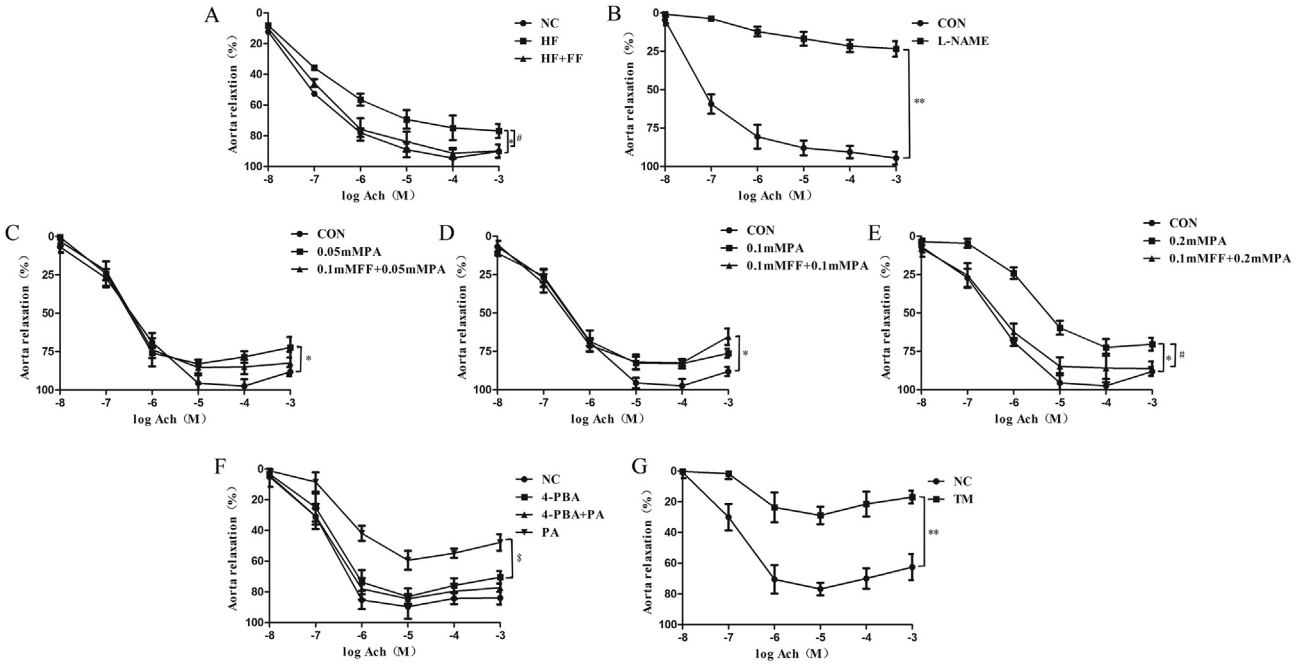

Fig. 2. Endothelium-dependent vasodilatation of thoracic aortas. A. Endothelium-dependent vasodilatation (EDV) in response to Ach, n = 6; B. EDV in response to Ach in the presence of *L*-NAME, n = 4; $^{*}p < 0.05$ vs. SCD; $^{\#}p < 0.05$ vs. HFD; $^{**}p < 0.01$ for *L*-NAME vs. control; C–E. EDV response to Ach, incubated with 0.05 mM, 0.1 mM and 0.2 mM PA respectively or preteated with 0.1 mM FF, n = 6; F. EDV response to Ach, pretreated with ER stress inhibitor 4-PBA, n = 6; G. EDV response to Ach, pretreated with ER stress inducer tunicamycin (TM). n = 6, $^{*}p < 0.05$, $^{**}p < 0.01$ for PA or TM vs. CON; $^{\#}p < 0.05$ for FF + PA vs. PA; $^{\$}p < 0.01$ for 4-PBA + PA vs. PA.

HFD-fed rats compared with SCD-fed rats, meanwhile FF treatment significantly ameliorated the impaired relaxation to Ach (Fig. 2A). When the endothelium was removed, relaxation to Ach was blunted compared with the intact vessels (data not show), indicated that this type of relaxation was dependent on intact endothelium. *L*-NAME was specific inhibitor of eNOS, incubation with *L*-NAME almost completely abolished the relaxation in aorta, suggested that EDV reaction to Ach mainly depended on eNOS activity (Fig. 2B). Our results indicated that chronic HFD feeding seriously impaired EDV, while FF treatment partly recovered the impaired EDV, this was consistent with the results of Omae et al. in isolated porcine retinal arteriole [13].

3.4. Effect of fenofibrate on impaired EDV induced by acute PA treatement

Previously we reported that ER stress induced by HCD feeding was possibly associated with endothelial dysfunction in mice aorta [4]. When incubated with PA, we found that 0.05 mM and 0.1 mM PA only influenced the relaxation in high-concentration of Ach (10^{-5} to 10^{-3} M), but when the concentration of PA was increased to 0.2 mM, the relaxation of aorta was reduced obviously. Pretreatment with 0.1 mM FF almost completely relieved the impaired EDV induced by 0.05–0.2 mM PA treatment (Fig. 2C–E).

To determine the relationship between ER stress and vascular reactivity, we examined EDV in the presence of ER stress inhibitor 4-PBA or inducer TM. Treatment with 4-PBA for 3 h alone had no effect on EDV, but pretreatment with 4-PBA followed by incubated with 0.2 mM PA could significantly relieved impaired EDV induced by PA (Fig. 2F). When incubated with TM for 90 min, the response to Ach was significantly reduced compared to that in vehicle (Krebs buffer) (Fig. 2G), indicated that FF and ER stress inhibitor all recovered damaged EDV induced by PA, and ER stress inducer TM could induce EDV.

3.5. Effect of fenofibrate on ER stress in thoracic aorta

RT-PCR analyses showed that HFD feeding induced ER stress in thoracic aorta by increasing CHOP (*Chop*) and GRP78 (*Grp78*) mRNA expression, meanwhile FF treatment alleviated HFD-induced ER stress (Fig. 3A, B). The changes of protein expression of GRP78 and CHOP were consistent with their respective mRNA expression (Fig. 3C, D). Further analyses of eNOS and p-eNOS indicated that p-eNOS was obviously reduced, meanwhile FF treatment significantly increased p-eNOS level (Fig. 3C, D). ERK and JNK are two downstream effectors of ER stress, specific activation of IRE1α pathway can lead to ERK and JNK activation [14,15]. The variation tendency of p-JNK relative to JNK and p-ERK relative to ERK were consistent with the changes of GRP78 and CHOP, suggested that JNK and ERK were activated by HFD feeding, while FF treatment could invert activated JNK and ERK through alleviation of GRP78 and CHOP (Fig. 3C, D).

3.6. Effect of FA on cell proliferation and NO production decreased by palmitic acid in MAEC

As shown in Fig. 4A–C, the proliferation of the MAEC was obviously suppressed by 0.1–1.0 mM PA, which exhibited a dose and time-dependent manner, the maximum inhibition was reached when treated with 1.0 mM PA for 24 h. Lu et al. also showed a dose-dependent reduction in cellular viability at doses of PA ranging from 100 μM to 400 μM [16]. FA significantly increased the cell viability suppressed by PA especially when the concentration was reached to 0.2 mM (Fig. 4C). NO was mainly produced by catalysis of eNOS, FA pretreatment obviously increased secretion of NO into supernatant which was decreased by PA treatment (Fig. 3E).

3.7. Effect of FA on PA-mediated ER stress and expression of eNOS in MAEC

ER stress in MAEC exposed to TM or PA for 24 h was determined by the gene expression of ER stress markers. TM or PA obviously

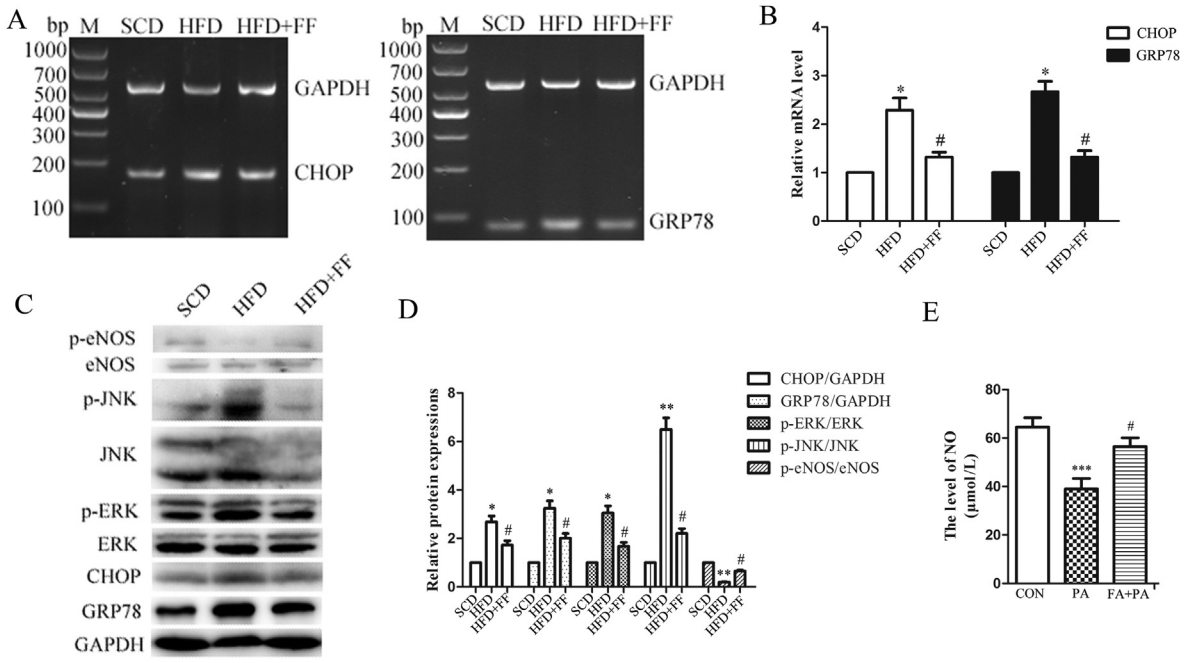

Fig. 3. Effects of fenofibrate on gene expression in thoracic aortas of HFD-fed female SD rats. A. Gene expression of *CHOP* and *GRP78* were analyzed by RT-PCR; B. Quantification of A; C. Western blot of eNOS, phosphorylation of eNOS(p-eNOS), JNK, phosphorylation of JNK (p-JNK), ERK, phosphorylation of ERK (p-ERK), CHOP and GRP78; D. Quantification of C. The experiment was repeated for three times. Data were presented as means ± S.D. $^{*}p < 0.05$ vs. SCD, $^{**}p < 0.01$ vs. SCD, $^{\#}p < 0.05$ vs. HFD; E. NO secretion from MAEC treated by PA or FA. Values are means ± SD, $^{***}p < 0.001$ vs. CON; $^{\#}p < 0.05$ vs. PA.

increased CHOP and GRP78 expression (Fig. 4D, E, H, I). IRE1α is a typical ER stress sensor, TM or PA also increased the mRNA expression of IRE1α and downstream spliced XBP1 (XBP-1s) mRNA, while FA or 4-PBA pretreatment obviously decreased the mRNA levels of IRE1α and XBP-1s (Fig. 4F, G).

The effect of PA on ER stress is specific as there had no changes in the expression of ER stress markers in MAEC incubated with vehicle and 4-PBA alone (Fig. 4D–F). Similar increase of p-ERK relative to ERK, p-JNK relative to JNK and decrease of p-eNOS relative to eNOS after incubation with TM or PA was exhibited in MAEC as in thoracic aorta, which were normalized after ER stress inhibition by 4-PBA or FA pretreatment (Fig. 4H, J).

4. Discussion

Obesity is a well-established cardiovascular risk factor, resulting in elevated circulating free fatty acids (FFA) and endothelial dysfunction, but so far few articles have reported endothelial dysfunction induced by PA and vascular protective effects of FF in hyperlipidemic animal models. In the present study, the endothelium-dependent vasodilatation (EDV) was investigated in thoracic aorta from FF-treated HFD-fed female rat model and normal SD female rats respectively (Fig. 2). This report also revealed a novel role of FF on EDV by ameliorating ER stress in rat endothelium and mouse aortic endothelial cells.

A standard HFD (D12451) was administered for 5 months to construct a hyperlipidemic female rat model successfully. The blood lipid indices were significantly elevated in HFD-fed female rats, and 2 months of FF treatment had obvious hypolipidemic activities except for TC levels. Chronic HFD feeding also deposited excess lipid in different vascular sites including intima, medium and extima, and caused irregular thickening in elastic fibers and impaired the integrity of vascular endothelium, while FF treatment significantly reverted these pathological changes (Fig. 1). To the best of our knowledge, this was the first study of FF treatment on elastic fibers of vessels in HFD-fed animal models.

It is well established that hyperlipidemia impairs endothelial function in experimental animals and humans as well [17,18]. NO is a typical vasodilator, opposite changes of NO level in serum of the HFD-fed and HFD + FF rats indicated the existence of endothelial dysfunction and improvement by FF treatment *in vivo* (Table 2). EDV in the isolated thoracic aorta from different groups showed that Ach-mediated vasodilation was severely impaired after chronic HFD feeding, meanwhile FF treatment obviously inverted the impaired EDV (Fig. 2A). Inhibition of NO synthesis by *L*-NAME almost completely abolished the EDV, suggesting that inactivation of endothelial NOS (eNOS) may play an important role in EDV (Fig. 2B). Some reports showed that FF only had a limited beneficial effect on vascular function [19,20], which were not consistent with our results, this maybe related with different diet components, different time and dose of FF treatment.

Chronic HFD feeding resulted in especially elevated PA (Table 2), so in order to confirm whether HFD-mediated impaired EDV was at least partly attributed to PA, the EDV experiment with PA and FF on the thoracic aorta from normal female SD rats was launched. The results showed that the EDV were obviously damaged by 0.2 mM PA in different all concentrations of Ach, while 0.1 mM FF pretreatment antagonized the damaged effect of PA on EDV (Fig. 2C–E). Then we hypothesized that the damaged EDV by PA was possibly related with PA-induced ER stress and alleviation of ER stress decreased damage of EDV. The results demonstrated that ER stress scavenger 4-PBA could antagonize damaged effect of PA on EDV (Fig. 2F), meanwhile ER stress inducer TM damaged EDV and showed stronger damage than 0.2 mM PA (Fig. 2G), strongly suggested that ER stress possibly participated in HFD and PA-induced damaged EDV.

There had seldom studies about mitigatory effect of FF on ER stress [21,22], even had report about increased ER stress by FF [23]. Our study showed that ER stress-related genes such as CHOP and

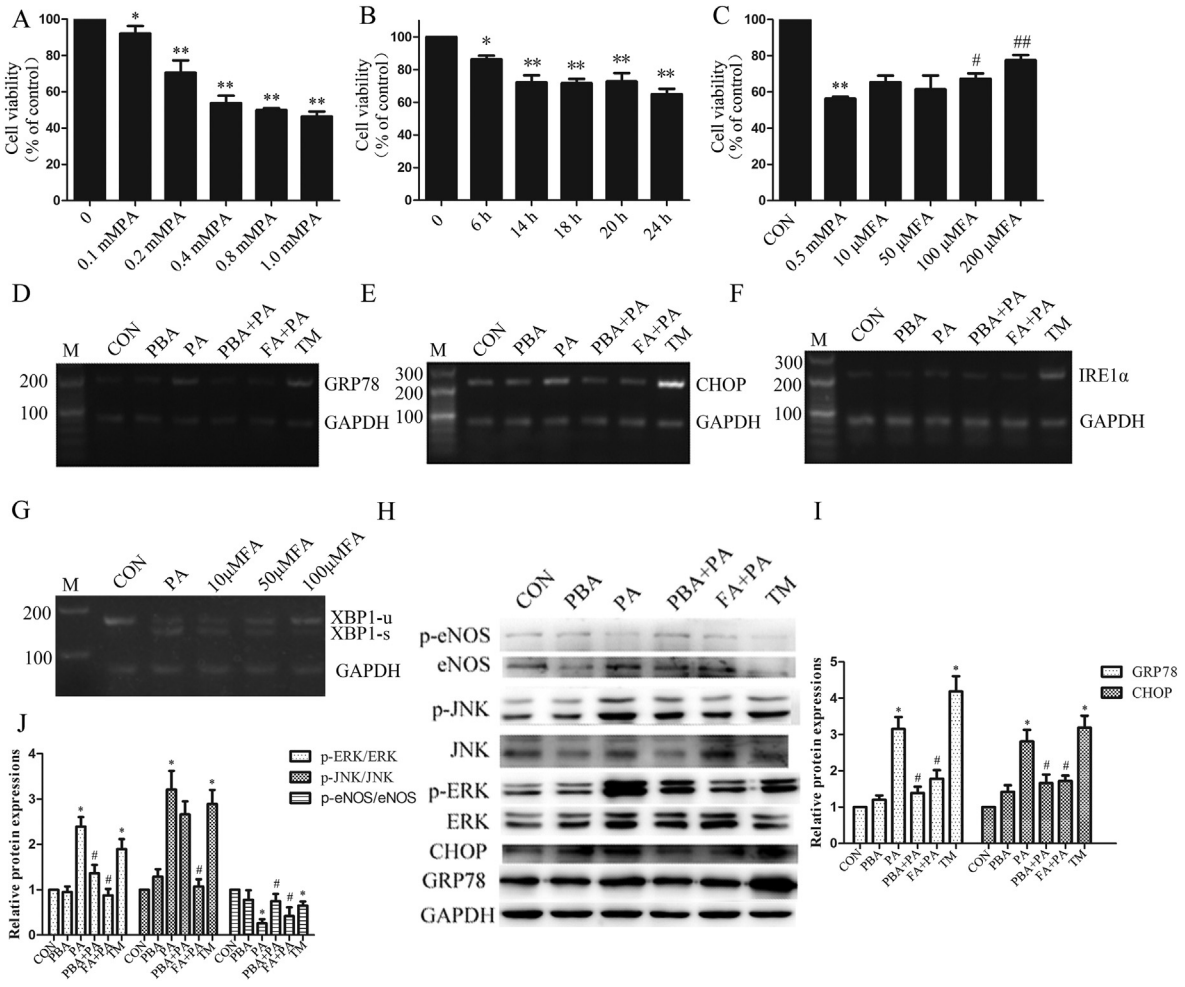

Fig. 4. Effects of FA on cell proliferation and gene expression in PA-treated MAEC. A. Dose effect of PA treatment; B. Time effect of PA treatment; C. Effect of FA pretreatment on PA-treated MAEC; D–G. Gene expression were analyzed by RT-PCR; H. Western blot of eNOS, phosphorylation of eNOS (p-eNOS), JNK, phosphorylation of JNK (p-JNK), ERK, phosphorylation of ERK (p-ERK), CHOP and GRP78. I–J. Quantification of H. Values are means ± S.D, $^{*}p < 0.05$, $^{**}p < 0.01$, vs. CON; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. PA.

GRP78, phosphorylation of ERK and JNK were obviously increased in HFD-induced thoracic aorta, while FF treatment obviously reverted ER stress and phosphorylation of ERK and JNK (Fig. 3). ER stress decreased phosphorylation of eNOS and activation, resulted in decreased NO formation and impaired EDV.

Then we confirmed the effects of PA and FA in primary MAEC. The proliferation of the MAEC was inhibited by PA and displayed in a time- and dose-dependent manner, while FA pretreatment increased proliferation and exhibited dose-dependent effect to some degree (Fig. 4A–C). Preliminary study from our laboratory demonstrated that PA increased protein expression of CHOP and GRP78 in primary MAEC [4], the present study demonstrated that expression of IRE1α and downstream XBP-1s were also significantly upregulated by PA, meanwhile FA pretreatment obviously decreased their expression (Fig. 4D–G). Protein expression of GRP78 and CHOP, phosphorylation of JNK and ERK1/2 were increased significantly, meanwhile phosphorylation of eNOS and NO levels in the supernatant was decreased obviously, FA pretreatment could partly recovered the impact of PA (Fig. 4H–J, 3E). The interesting discovery was that when 4-PBA was added into medium along with PA, it reverted the influence of PA on phosphorylation of JNK, ERK and eNOS, strongly suggested that PA exerted lipotoxicity by inducing ER stress and FA antagonized effect of PA at least partly through alleviating ER stress in primary aortic endothelial cells. These suggestions should be confirmed via further inhibitor and RNAi experiments.

In conclusion, high-fat intake and palmitic acid treatment induced endothelial dysfunction by increasing ER stress and decreasing phosphorylation of eNOS. Fenofibrate restored endothelium-dependent vasodilation via inhibiting ER stress and increasing phosphorylation of eNOS, thus provides possible clinical usage on macrovascular diseases in future.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by research grants from Anhui Medical University for Scientific Research of BSKY (XJ201013), Fund of Anhui Natural Science Foundation of China (1208085MH168 and 1308085MH154).

References

[1] [a] C. Koumaras, N. Katsiki, V.G. Athyros, A. Karagiannis, Metabolic syndrome and arterial stiffness: the past, the present and the future, Cardiovasc. Med. 14

(2013) 687–689;
[b] S. Mottillo, K.B. Filion, J. Genest, L. Joseph, L. Pilote, P. Poirier, S. Rinfret, E.L. Schiffrin, M.J. Eisenberg, The metabolic syndrome and cardiovascular risk a systematic review and meta-analysis, J. Am. Coll. Cardiol. 56 (2010) 1113–1132.
[2] D. Tian, Y. Qiu, Y. Zhan, X. Li, X. Zhi, X. Wang, L. Yin, Y. Ning, Overexpression of steroidogenic acute regulatory protein in rat aortic endothelial cells attenuates palmitic acid-induced inflammation and reduction in nitric oxide bioavailability, Cardiovasc. Diabetol. 11 (2012) 144.
[3] M. Shen, L. Wang, G. Yang, L. Gao, B. Wang, X. Guo, C. Zeng, Y. Xu, L. Shen, K. Cheng, Y. Xia, X. Li, H. Wang, L. Fan, X. Wang, Baicalin protects the cardiomyocytes from ER stress-induced apoptosis: inhibition of CHOP through induction of endothelial nitric oxide synthase, PLoS One 9 (2014) e88389.
[4] Y. Lu, L. Qian, Q. Zhang, B. Chen, L. Gui, D. Huang, G. Chen, L. Chen, Palmitate induces apoptosis in mouse aortic endothelial cells and endothelial dysfunction in mice fed high-calorie and high-cholesterol diets, Life Sci. 92 (2013) 1165–1173.
[5] M. Bosma, D.H. Dapito, Z. Drosatos-Tampakaki, N. Huiping-Son, L.S. Huang, S. Kersten, K. Drosatos, I.J. Goldberg, Sequestration of fatty acids in triglycerides prevents endoplasmic reticulum stress in an in vitro model of cardiomyocyte lipotoxicity, Biochim. Biophys. Acta (BBA) Mol. Cell. Biol. L 1841 (2014) 1648–1655.
[6] K. McKeage, G.M. Keating, Fenofibrate: a review of its use in dyslipidaemia, Drugs 71 (2011) 1917–1946.
[7] H. Shi, D.J. Clegg, Sex differences in the regulation of body weight, Physiol. Behav. 97 (2009) 199–204.
[8] R. Arakawa, N. Tamehiro, T. Nishimaki-Mogami, K. Ueda, S. Yokoyama, Fenofibric acid, an active form of fenofibrate, increases apolipoprotein A-I-mediated high-density lipoprotein biogenesis by enhancing transcription of ATP-binding cassette transporter A1 gene in a liver X receptor-dependent manner, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 1193–1197.
[9] L.D. Han, J.F. Xia, Q.L. Liang, Y. Wang, Y.M. Wang, P. Hu, P. Li, G.A. Luo, Plasma esterified and non-esterified fatty acids metabolic profiling using gas-chromatography mass spectrometry and its application in the study of diabetic mellitus and diabetic nephropathy, Anal. Chim. Acta 689 (2011) 85–91.
[10] C. Napoli, F.P. D'Armiento, F.P. Mancini, A. Postiglione, J.L. Witztum, G. Palumbo, W. Palinski, Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal streak formation occurs in human fetal aortas and is greatly enhanced by maternal monocyte recruitment formation occurs in human fetal aortas and is greatly enhanced by maternal monocyte recruitment, J. Clin. Invest. 100 (1997) 2680–2690.
[11] C.N. Battlehner, E.G. Caldini, J.C. Pereira, E.H. Luque, G.S. Montes, How to measure the increase in elastic system fibres in the lamina propria of the uterine cervix of pregnant rats, J. Anat. 203 (2003) 405–418.
[12] J. Dou, H. Li, X. Ma, M. Zhang, Q. Fang, M. Nie, Y. Bao, W. Jia, Osteocalcin attenuates high fat diet-induced impairment of endothelium-dependent relaxation through Akt/eNOS-dependent pathway, Cardiovasc. Diabetol. 13 (2014) 74.
[13] T. Omae, T. Nagaoka, I. Tanano, T. Kamiya, A. Yoshida, Fenofibrate, an anti-dyslipidemia drug, elicits the dilation of isolated porcine retinal arterioles: role of nitric oxide and AMP-activated protein kinase, Invest. Ophthalmol. Vis. Sci. 53 (2012) 2880–2886.
[14] M.J. Jurczak, A.H. Lee, F.R. Jornayvaz, H.Y. Lee, A.L. Birkenfeld, B.A. Guigni, M. Kahn, V.T. Samuel, L.H. Glimcher, G.I. Shulman, Dissociation of inositol-requiring enzyme (IRE1α)-mediated c-Jun N-terminal kinase activation from hepatic insulin resistance in conditional X-box-binding protein-1 (XBP1) knock-out mice, J. Biol. Chem. 287 (2012) 2558–2567.
[15] A. Kato, T. Okura, C. Hamada, S. Miyoshi, H. Katayama, J. Higaki, R. Ito, Cell stress induces upregulation of osteopontin via the ERK pathway in type II alveolar epithelial cells, PLoS One 9 (2014) e100106.
[16] Z.H. Lu, Y.M. Mu, B.A. Wang, X.L. Li, J.M. Lu, J.Y. Li, C.Y. Pan, T. Yanase, H. Nawata, Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell, Biochem. Biophys. Res. Commun. 303 (2003) 1002–1007.
[17] E.B. Okon, A.W. Chung, H. Zhang, I. Laher, C. van Breemen, Hyperglycemia and hyperlipidemia are associated with endothelial dysfunction during the development of type 2 diabetes, Can. J. Physiol. Pharmacol. 85 (2007) 562–567.
[18] D. Erdogan, H. Gullu, M. Caliskan, O. Ciftci, S.T. Baycan, M. Bilgi, T. Ulus, S. Kulaksizoglu, H. Muderrisoglu, Fasting hypertriglyceridaemia increases carotid intima-media thickness and impairs coronary microvascular functions in non-obese middle aged women but not in men, Heart 92 (2006) 259–260.
[19] X. Zhao, J.E. Quigley, J. Yuan, M.H. Wang, Y. Zhou, J.D. Imig, PPAR-alpha activator fenofibrate increases renal CYP-derived eicosanoid synthesis and improves endothelial dilator function in obese Zucker rats, Am. J. Physiol. Heart Circ. Physiol. 290 (2006) H2187–H2195.
[20] E.K. Naderali, S. Fatani, G. Williams, Fenofibrate lowers adiposity and corrects metabolic abnormalities, but only partially restores endothelial function in dietary obese rats, Atherosclerosis 177 (2004) 307–312.
[21] S.M. Rahman, I. Qadri, R.C. Janssen, J.E. Friedman, Fenofibrate and PBA prevent fatty acid-induced loss of adiponectin receptor and pAMPK in human hepatoma cells and in hepatitis C virus-induced steatosis, J. Lipid Res. 50 (2009) 2193–2202.
[22] S. Miranda, A. González-Rodríguez, M. García-Ramírez, J. Revuelta-Cervantes, C. Hernández, R. Simó, Á.M. Valverde, Beneficial effects of fenofibrate in retinal pigment epithelium by the modulation of stress and survival signaling under diabetic conditions, J. Cell. Physiol. 227 (2012) 2352–2362.
[23] S.M. Chan, R.Q. Sun, X.Y. Zeng, Z.H. Choong, H. Wang, M.J. Watt, J.M. Ye, Activation of PPARα ameliorates hepatic insulin resistance and steatosis in high fructose-fed mice despite increased endoplasmic reticulum stress, Diabetes 62 (2013) 2095–2105.