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Research Paper

Suppression of inflammatory response and endothelial nitric oxide synthase downregulation in hyperlipidaemic C57BL/6J mice by eugenosedin-A

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Abstract

Objectives Eugenosedin-A has been found to ameliorate high-fat diet (HFD)-induced hyperglycaemia and hyperlipidaemia in C57BL/6J mice. This study aimed to investigate the mechanisms of action of eugenosedin-A on endothelial function and inflammation in hyperlipidaemic mice.

Methods C57BL/6J mice were randomly divided into two control groups and two treatment groups. The control mice received either a regular diet or HFD, and the treatment groups were fed HFD with either 5 mg/kg eugenosedin-A or atorvastatin for eight weeks.

Key findings Mice fed a HFD had higher concentrations of nitrate (NO) but not prostaglandin E_2 (PGE_2), increased tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) mRNA and inducible nitric oxide synthase (iNOS) proteins, but decreased endothelial nitric oxide synthase (eNOS) proteins. HFD-induced upregulation of iNOS is associated with p38, extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), PI3K and Akt/IKKα/p65. Eugenosedin-A and atorvastatin reduced HFD-induced TNF-α and IFN-γ mRNA, NO generation, upregulation of iNOS protein, and down-regulation of eNOS protein. Both agents inhibited p38, ERK, JNK and Akt/IKKα/p65 protein levels in the aorta. However, eugenosedin-A did not significantly reduce p38 in the liver.

Conclusions Our results showed an association between obesity-induced inflammation and altered levels of TNF-α, IFN-γ, p38, ERK, JNK and Akt/IKKα/p65. Eugenosedin-A, like atorvastatin, could inhibit p38, ERK, JNK, Akt/IKKα/p65 proteins, as well as TNF-α and IFN-γ mRNA during the regulation of the obesity-induced inflammatory process.

Keywords atorvastatin; eNOS; eugenosedin-A; hyperlipidaemia; inflammation

Introduction

Cardiovascular complications are the main causes of morbidity and mortality in diabetic and obese patients.[1] Several mechanisms are involved in the development of these complications, including inflammation and oxidation stress.[2,3] Hypercholesterolaemia increases reactive oxygen species (ROS) by stimulating polymorphonuclear leucocytes and activating endothelial cells and has been associated with increased levels of tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ).[4,5] There is an increase in inducible nitric oxide synthase (iNOS) mRNA as well as protein expression and activity in hypercholesterolaemia.[6] These cytokines and iNOS can damage endothelial cells indirectly by stimulating the expression a variety of genes involved in inflammatory responses. One key regulator of these genes is nuclear factor-κB (NF-κB). NF-κB is activated by different mitogen-activated protein kinases (MAPKs) and is involved in the expression of cytokines.[7] Atherosclerosis develops within vascular lesions that have focally expressed acute inflammatory cytokines such as TNF-α and interleukin-1 (IL-1). These genes in turn activate such MAPKs as Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun-N-terminal kinase (JNK) and p38.[8–10]

NF-κB can reduce endothelial production of nitric oxide (NO) by inhibiting the formation of endothelial nitric oxide synthase (eNOS). If eNOS is damaged, then there is a reduction in NO production and a loss of vasodilatory, anti-inflammatory and

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antiproliferative properties, leading to endothelial dysfunction, atherosclerosis and restenosis.[11] Hypercholesterolaemia increases the expression of cytokines such as TNF-α and IFN-γ, which are known to activate granulocytes to generate ROS.[12,13] It increases the levels of ROS and nitrogen species, which in turn reduces NO availability and impairs endothelial vasomotor function.[14]

In previous studies, we found eugenosedin-A, a 5-$HT_{1B/2A}$ and $\alpha_1/\alpha_2/\beta_1$-adrenergic blocker, to be capable of reducing inflammation and scavenging free radicals.[15,16] We found that, like atorvastatin, it reduced weight gain and corrected obesity-associated hyperglycaemia, hyperinsulinaemia, hyperlipidaemia, and lipid peroxidation in mice fed high-fat diets (HFD).[17] However, we do not know if eugenosedin-A, like atorvastatin, can prevent HFD-induced inflammation. To find out, we have measured the effects of eugenosedin-A and atorvastatin on plasma nitrate and prostaglandin E_2 (PGE_2), and their effect on expression of TNF-α and IFN-γ mRNA, MAPKs, iNOS, cyclooxygenase-2 (COX-2) and eNOS in the liver and aorta of mice fed a HFD. In this study, we first demonstrated that eugenosedin-A had novel effects on HFD-induced inflammation, which were due to antagonism of serotonergic and α/β-adrenergic receptors. So far, agents with combined serotonergic and α/β-adrenergic antagonistic responses for the prevention or control of hyperlipidaemia-induced inflammation have been little described. This unique mechanism of eugenosedin-A was in contrast to that of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor atorvastatin in the control of hyperlipidaemia-induced inflammation.

Materials and Methods

Animals

Female mice (C57BL/6J strain) were obtained by the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and housed under constant temperature and illumination (light between 7:30 and 19:30 h). Water and a regular diet were freely available. After acclimatization, the six-week-old mice were randomly divided into two control groups and two treatment groups. For eight weeks, one control group ($n = 8$) was fed a regular diet and the other control group ($n = 8$) was fed a HFD (cat No. 58G9, TestDiet, Richmond, USA). The HFD contained 60% fat, 21.4% carbohydrates and 18.6% protein. Both treatment groups were fed the HFD, but one group was supplemented with 5 mg/kg eugenosedin-A whilst the other group was supplemented with 5 mg/kg atorvastatin. For all groups, body weight was measured weekly. At the end of the study blood was collected, and the liver and thoracic aorta excised from all mice. The blood was used for biochemical assay. Tissues were stored in buffer solution at −80°C until analysis. This study was approved by the Animal Care and Use Committee of Kaohsiung Medical University.

Drugs and chemicals

Atorvastatin was purchased from Sigma Chemical Co. (St Louis, MO, USA). Eugenosedin-A, 4-(2-hydroxy-3-[1-(2-chlorophenyl-piperazinyl)]-propoxy)-3-methoxy-1-propylenyl-benzene, was synthesized in our laboratory by combining isoeugenol-based oxypropanolamine and 2-chloro-1-piperazinyl benzene (CPB), and was purified by column chromatography and recrystallized with ethanol to achieve > 95% purity.[15] Eugenosedin-A and other agents were solubilized in 5% absolute alcohol. Further dilutions were made in distilled water.

Quantification of mRNA levels in the liver and aorta

Total cellular RNA was extracted by using RNA Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The expression of liver and aorta subgenomic RNA were detected by quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) with primers corresponding to TNF-α gene; forward primer: 5′-TGA,GCA,CAG,AAA,GCA,TGA,TCC,GC-3′ and reverse primer: 5′-GGT,GGT,TTG,CTA,CGA,CGT,GGG,C-3′, and IFN-γ gene; forward primer: 5′-TAC,TGC,CAC,GGC,ACA,GTC,ATT,GAA-3′ and reverse primer: 5′-GCA,GCG,ACT,CCT,TTT,CCG,CTT,CCT-3′. Each sample was normalized by an endogenous reference gene β-actin; forward primer: 5′-ACAGCTGAGAGGGAAATCGTG-3′ and reverse primer: 5′-CTAGGAGCCAGGGCAGTAATCT-3′. The cDNA quantification was measured by the ABI Step One Real-Time PCR-System (ABI Warrington, UK).

Western blot analysis of MAPKs, iNOS, COX-2 and eNOS expression

Tissue samples (liver and thoracic aorta) were obtained and frozen at −80°C before assay. Frozen tissues were homogenized on ice with a polytron PT 3000 homogenizer (Kinematic AG, Littau, Switzerland) in a buffer composed of (mM): Tris-HCl 50, EDTA 0.1, EGTA 0.1, 2-mercaptoethanol 12, and phenylmethylsulphonyl fluoride 1 (pH 7.4). The homogenized tissues were centrifuged at 10 000*g* for 30 min and the supernatants were stored at −80°C until further analysis. Samples of tissue homogenates were used for the protein assay (Bio-Rad protein assay reagent) and Western blot analysis. Tissue homogenates containing 20 µg protein were reduced and separated on 7.5% SDS-PAGE gel using Phast-System with PhastGel (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The membranes were blocked with 1% bovine serum albumin in Tris buffer solution (TBS) containing 0.1% Tween-20 for 2 h and then incubated with anti-mouse Tak, Ask, MEK3/6, p38, Src, Ras, MEK1/2, ERK1, Rac, MEK4, JNK1, PI3K, IKKα, Akt, p65, iNOS, COX-2 and eNOS antibody (Santa Cruz Biotechnology, CA, USA; 1 : 500 dilution) in TBS containing 0.1% Tween-20 for 2 h. The membrane was washed and finally incubated with a 1 : 1000 dilution of anti-mouse IgG conjugated to horseradish antibody for 2 h. After successive washings, the immunocomplexes were developed using an enhanced horseradish peroxide/luminol chemiluminescence reaction (ECL Western blotting detection reagents, GE Healthcare Bio-Sciences Corp.) and exposed to X-ray film for 10 min. The relative expression of those proteins in each tissue was quantified by

densitometric scanning of the Western blots using Image-pro plus software (Media Cybernetics, MD, USA) as described previously.[17]

Determination of plasma nitrate and PGE_2

Blood samples were collected from a catheter placed in the carotid artery and centrifuged at 7200*g* for 10 min to obtain the plasma to measure nitrate (Cayman Chemical Co., Ann Arbor, MI, USA) and PGE_2 (R&D Systems, Minneapolis, MN, USA) levels using an enzyme-linked immunoadsorbent assay (ELISA) kit as described previously.[18] The limit of detection of the kits for nitrate and PGE_2 were 2.5 μM and 16.0~41.1 pg/ml, respectively.

Statistical evaluation of data

Results are expressed as mean ± SE. Statistical differences were determined by independent and paired Student's *t*-test in unpaired and paired samples, respectively. Whenever a control group was compared with more than one treatment group, we used one-way analysis of variance or two-way repeated measures analysis of variance. If a significant difference was found, we used Dunnett's or Student-Newman-Keuls test for further analysis. A P-value < 0.05 was considered significant in all experiments. Analysis of data and plotting of figures was done using SigmaStat version 2.03 and SigmaPlot version 8.0 (Systat Software, Point Richmond, CA, USA).

Results

TNF-α and IFN-γ mRNA levels in the liver and aorta

The RT-qPCR data showed that TNF-α and IFN-γ mRNA levels were significantly enhanced in the liver and aorta in the HFD group. Eugenosedin-A and atorvastatin could clearly diminish the mRNA levels of TNF-α and IFN-γ in hyperlipidaemic mice (Figure 1).

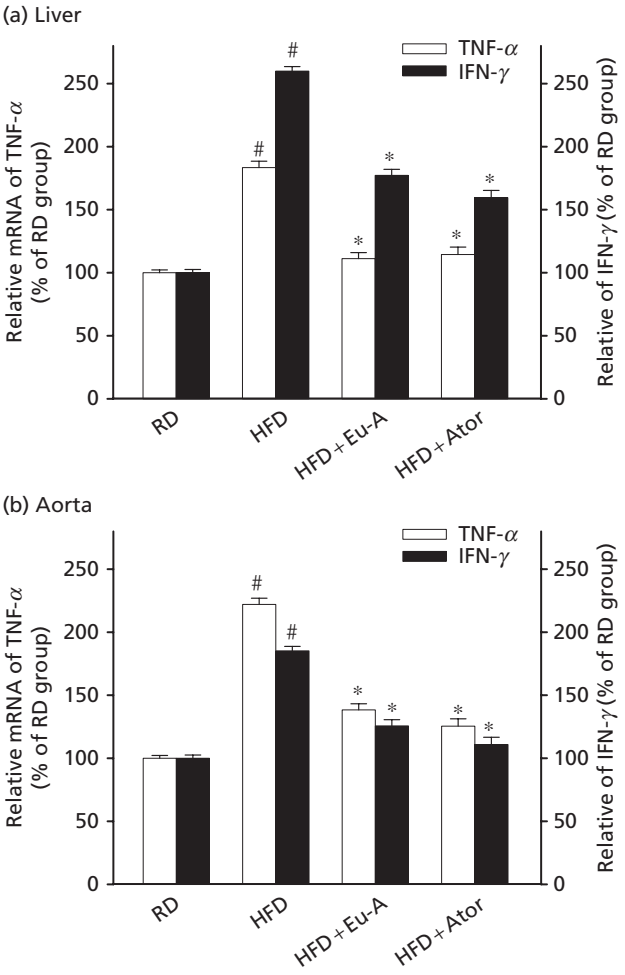

Figure 1 Effects of eugenosedin-A on tumour necrosis factor-α and interferon-γ mRNA expression in liver and aorta in high-fat diet fed mice. Mice were fed a regular diet (RD), a high-fat diet (HFD), or a HFD with either 5 mg/kg eugenosedin-A (Eu-A) or atorvastatin (Ator) for eight weeks. Each value represents the mean ± SE ($n = 8$). #$P < 0.05$ vs RD; *$P < 0.05$ vs HFD. TNF-α, tumour necrosis factor-α; IFN-γ, interferon-γ.

p38 protein expression in liver and aorta

The HFD-fed group had a significant increase in p38 and its mitogen-activated protein kinases Tak, Ask and MEK3/6 in liver and aorta, compared with the group fed a regular diet. Eugenosedin-A slightly decreased but atorvastatin clearly decreased Tak, Ask, MEK3/6 and p38 protein levels in HFD-fed mice in liver. However, both agents markedly reduced the expression of Tak, Ask, MEK3/6 and p38 protein in the aorta (Figure 2).

ERK protein expression in liver and aorta

The HFD-fed group had a significant increase in ERK1 and its mitogen-activated protein kinases Src, Ras and MEK1/2 in liver and aorta, compared with the group fed a regular diet. Eugenosedin-A and atorvastatin decreased Src, Ras, MEK1/2 and ERK1 protein levels in HFD-fed mice in liver and aorta (Figure 3).

JNK protein expression in liver and aorta

The HFD group had significant increases in Rac, MEK4/7 and JNK1 protein levels in the liver and aorta. Eugenosedin-A and atorvastatin diminished the expression of those proteins (Figure 4).

PI3K, Akt, IKKα and p65 protein expression in liver and aorta

The protein expression of PI3K, Akt, IKKα and p65 were higher in the liver and aorta in the HFD group than in the regular diet group. Eugenosedin-A and atorvastatin markedly reduced Akt, IKKα and p65 protein levels, but not PI3K (Figure 5).

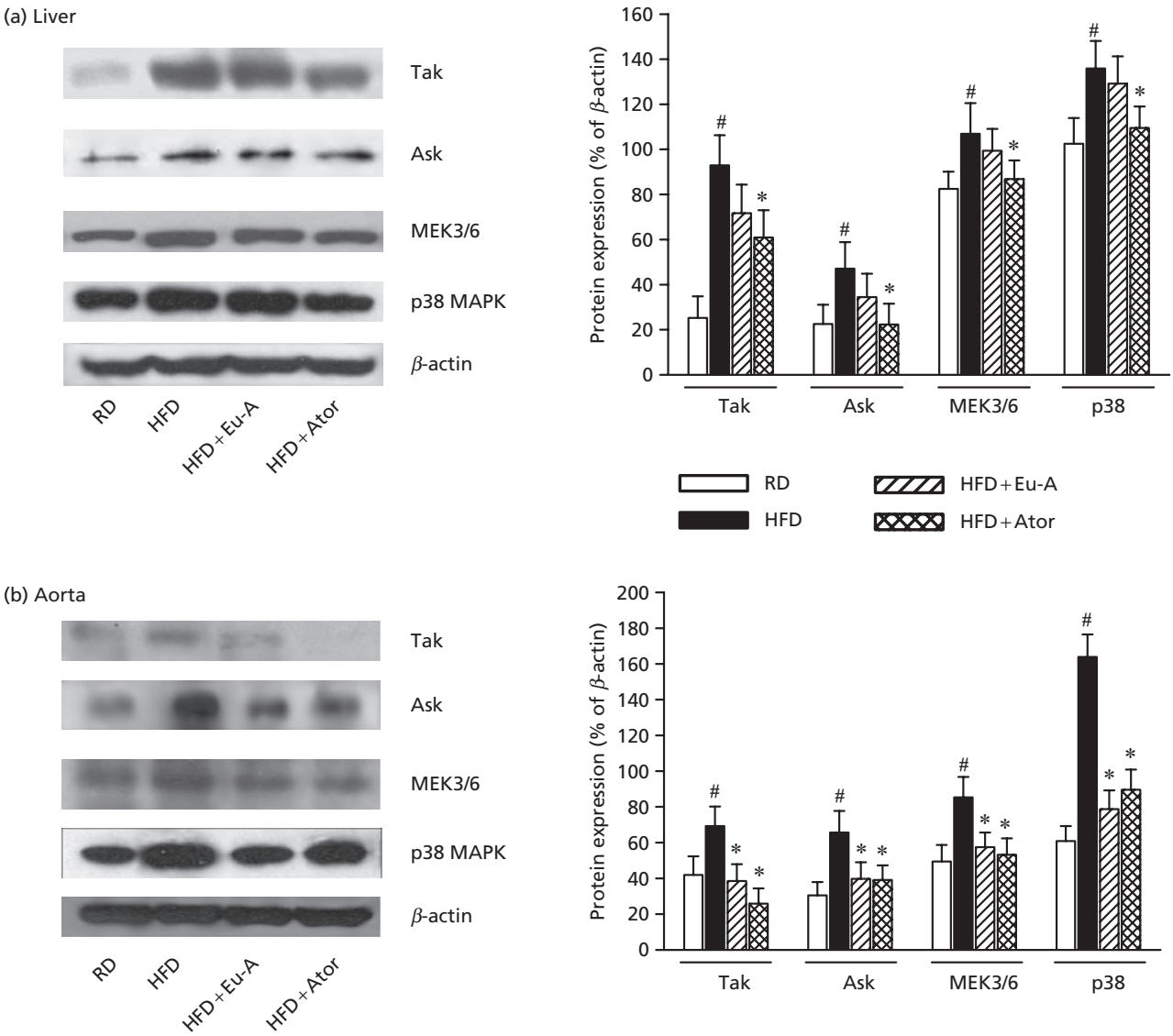

Figure 2 Effects of eugenosedin-A on p38 MAPK pathway protein expression in liver and aorta in high-fat diet fed mice.
Mice were fed a regular diet (RD), a high-fat diet (HFD), or a HFD with either 5 mg/kg eugenosedin-A (Eu-A) or atorvastatin (Ator) for eight weeks. Each value represents the mean ± SE ($n = 8$). #$P < 0.05$ vs RD; *$P < 0.05$ vs HFD.

iNOS, COX-2 protein expression in liver and aorta, and eNOS protein expression in aorta

iNOS protein levels were higher in the liver and aorta of the HFD group than in the regular diet group, while neither control group was found to have a significant difference in COX-2 protein level (data not shown). Eugenosedin-A and atorvastatin patently reduced iNOS protein levels in liver and aorta (Figure 6). The eNOS protein level decreased in the HFD group, compared with the group fed the regular diet. Eugenosedin-A and atorvastatin clearly recovered eNOS proteins in aorta (Figure 6).

Plasma nitrate and PGE_2 levels

The level of nitrate concentration significantly increased in the HFD group (149.68 ± 9.93 μM) compared with the one fed a regular diet (55.33 ± 3.12 μM). Plasma nitrate concentration in the eugenosedin-A- (72.03 ± 2.18 μM) or atorvastatin-treated group (81.37 ± 5.07 μM) was significantly lower than that of the HFD group. The PGE_2 levels in the regular diet group and HFD group were 701.5 ± 14.2 and 725.7 ± 20.6 pg/ml, respectively. However, PGE_2 levels showed no significant changes in either the eugenosedin-A- (713.6 ± 28.3 pg/ml) or the atorvastatin (699.3 ± 26.8 pg/ml)-treated groups.

Discussion

Previously, we demonstrated that eugenosedin-A was able to reduce glucose and lipid levels and indicated that in the future it may be used to improve obesity-related hyperglycaemia, hyperinsulinaemia, and hyperlipidaemia and scavenge free

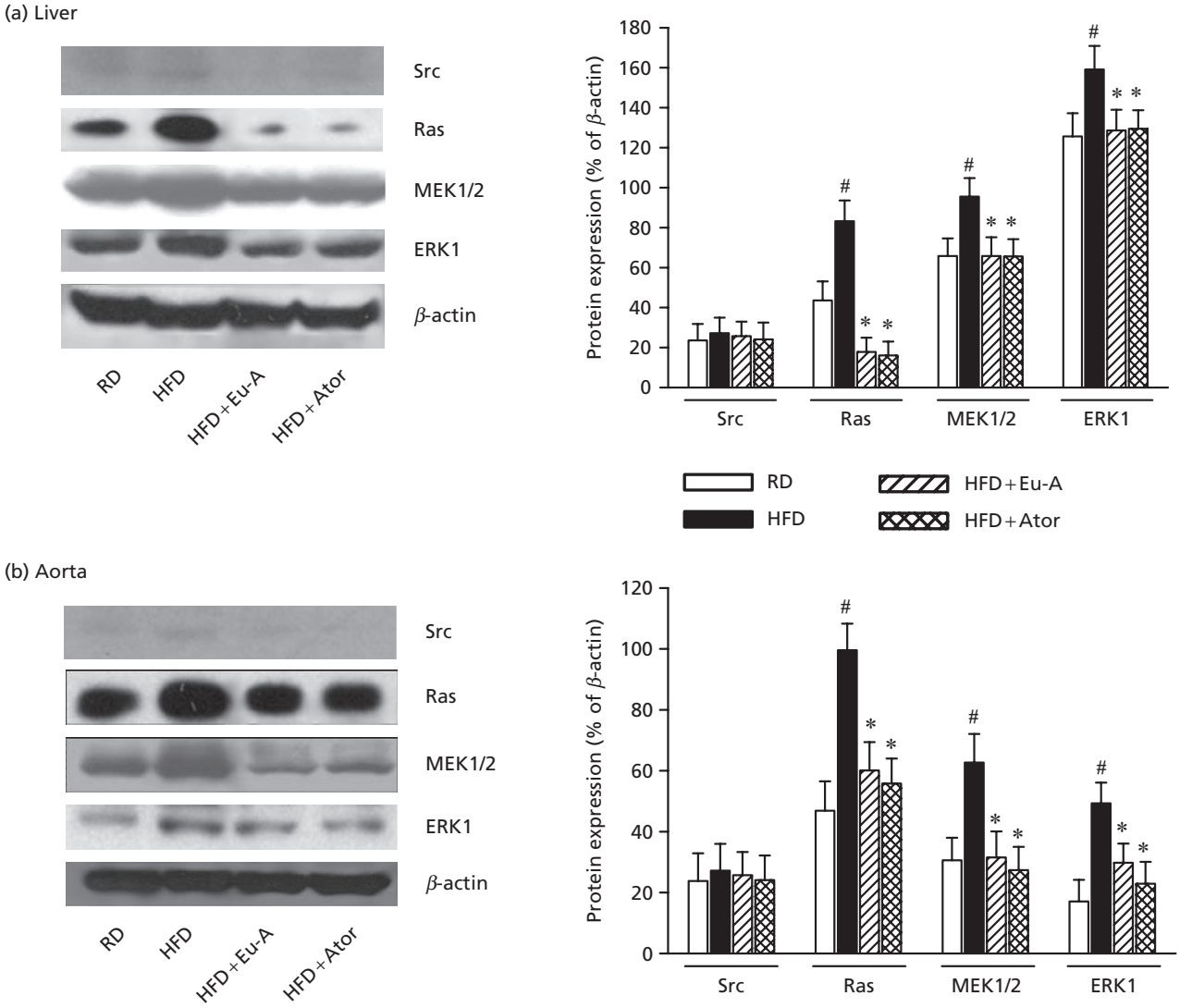

Figure 3 Effects of eugenosedin-A on ERK pathway protein expression in liver and aorta in high-fat diet fed mice.
Mice were fed a regular diet (RD), a high-fat diet (HFD), or a HFD with either 5 mg/kg eugenosedin-A (Eu-A) or atorvastatin (Ator) for eight weeks. Each value represents the mean ± SE ($n = 8$). #$P < 0.05$ vs RD; *$P < 0.05$ vs HFD.

radicals, which would result in the reduction of obesity-induced lipid peroxidation.[17] In this study, we have provided further evidence that eugenosedin-A reduced protein levels of p38, ERK, JNK, Akt/IKKα/p65 and mRNA expression of TNF-α and IFN-γ in the liver and aorta, and reduced nitrate in the plasma of mice with hyperlipidaemia-induced inflammation. However, we did not find a good correlation between hyperlipidaemia and PGE_2. We found a decrease in all ERK, JNK, p65 protein, iNOS, and TNF-α, IFN-γ mRNA in the liver of mice fed the HFD.

Hypercholesterolaemia was induced in mice by increasing their intake of dietary lipids, a major cause of atherogenesis. Atherogenesis requires inflammatory components, which were thought to make the progression of the disease.[19] The liver plays a key role in the inflammatory response evoked by dietary constituents.[20] These findings suggested that nutritional cholesterol may contribute to the evolution of the inflammatory component of atherogenesis. Steinberg[19] supposed that proatherogenic inflammatory factors originated at least in part from the liver, and he found that cytokines TNF-α and IFN-γ and the transcriptional regular NF-κB played central roles in the development of cholesterol-induced liver inflammation. In addition, increases in mRNA and protein expression and iNOS activity have been found in obese mice or hypercholesterolaemic rabbits.[6,21] Taken together, these findings suggested that hyperlipidaemia could induce inflammation in the liver. However, in a previous animal study, we found no relationship between hyperlipidaemia and alanine aminotransferase (ALT) and aspartate aminotransferase (AST), though this lack of such a relationship could be due to the short length of time studied (eight weeks).[17] In this study, we found that eugenosedin-A

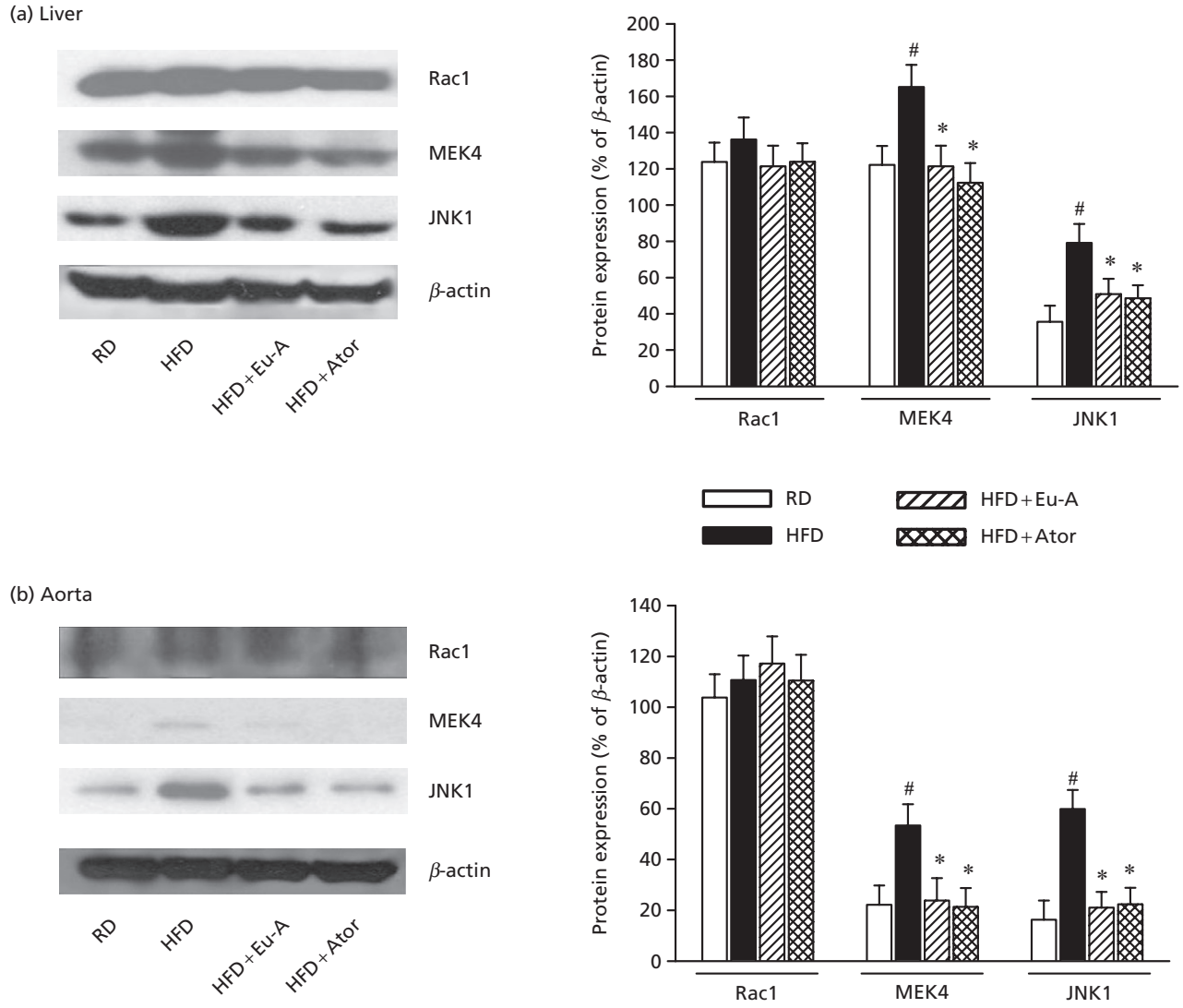

Figure 4 Effects of eugenosedin-A on JNK pathway protein expression in liver and aorta in high-fat diet fed mice.
Mice were fed a regular diet (RD), a high-fat diet (HFD), or a HFD with either 5 mg/kg eugenosedin-A (Eu-A) or atorvastatin (Ator) for eight weeks. Each value represents the mean ± SE ($n = 8$). #$P < 0.05$ vs RD; *$P < 0.05$ vs HFD.

significantly reduced the upstream of ERK, JNK and p65 pathways, and iNOS protein expression in the liver of HFD mice, but p38 and PI3K were not significantly reduced. Eugenosedin-A and atorvastatin significantly decreased the mRNA expressions of TNF-α and IFN-γ. Together these findings suggested that like atorvastatin, eugenosedin-A could markedly ameliorate hyperlipidaemia-induced liver inflammation.

The liver is thought to play a role in inflammatory responses, including proatherogenic inflammatory responses, evoked by dietary constituents.[19,20] In addition, inflammatory cell recruitment and activation are important events in the pathological changes in blood vessels. Different mediators and blood cell populations have been found to be involved in hypercholesterolaemia-induced inflammatory responses.[22] For example, proinflammatory mediators such as TNF-α and IFN-γ have been reported to be able to upregulate NF-κB, a gene involved in inflammatory responses.[23] NF-κB can be activated also by p38, ERK1/2 and JNK leading to subsequent proatherogenic changes.[24] At the same time, iNOS protein expression might increase. Angiogenic actions of NO are known to be able to accelerate vascular dysfunction under hypercholesterolaemia.[6] In this study, we found that hyperlipidaemia elicited the inflammatory response in the aorta. The mRNA expressions of TNF-α, IFN-γ and protein levels of p38, ERK, JNK, p65 and iNOS increased in the aorta. Therefore, we suggest that hyperlipidaemia could provoke inflammation in the aorta. Eugenosedin-A and atorvastatin clearly inhibited the upstream of p38, ERK, JNK and p65 pathways, and iNOS protein expression in the HFD mice aorta. Both agents also decreased the mRNA expressions of TNF-α and IFN-γ.

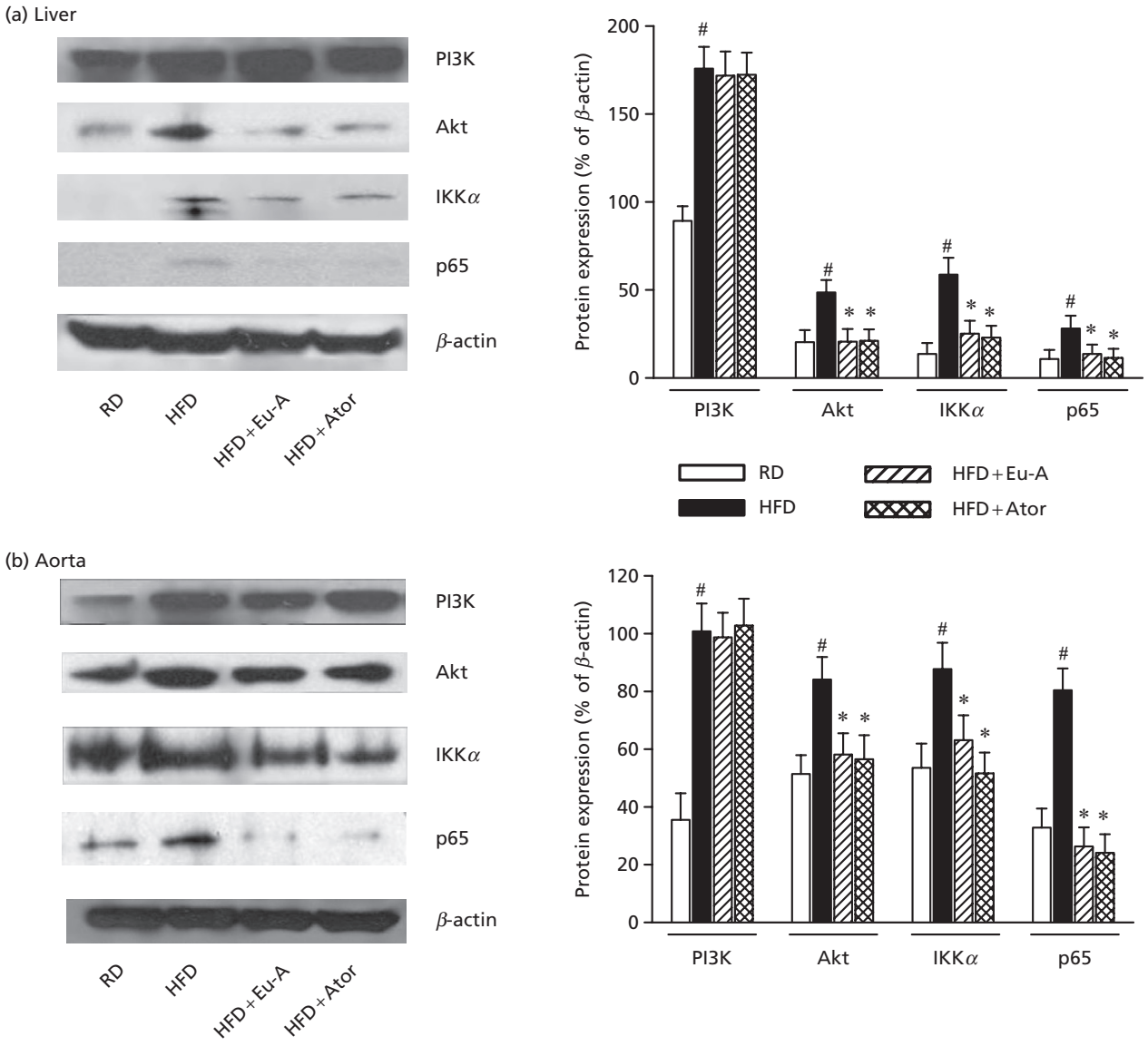

Figure 5 Effects of eugenosedin-A on p65 pathway protein expression in liver and aorta in high-fat diet fed mice.
Mice were fed a regular diet (RD), a high-fat diet (HFD), or a HFD with either 5 mg/kg eugenosedin-A (Eu-A) or atorvastatin (Ator) for eight weeks. Each value represents the mean ± SE ($n = 8$). #$P < 0.05$ vs RD; *$P < 0.05$ vs HFD.

Additionally, both treatment groups decreased the plasma NO concentrations. Eugenosedin-A and atorvastatin were found to improve hyperlipidaemia-induced aorta inflammation and NO accumulation.

Endothelial cells are damaged by stimulating the expression of a variety genes involved in inflammatory responses.[9] Oxidants and cytokines, such as TNF-α and IFN-γ, have been implicated in the endothelial cell dysfunction and atherosclerotic lesion formation observed in arteries of hypercholesterolaemic animals. Cytokines also reduced transcription and increased instability of eNOS mRNA.[25] Kawashima and Yokoyama[26] demonstrated that eNOS became dysfunctional and produced superoxide rather than NO in hyperlipidaemia and atherosclerosis. Although there were several factors underlying the reduced endothelium-dependent relaxation, the most important factor was derangements of the eNOS/NO pathway, which occurred with reduced activity and expression of eNOS, decreased sensitivity to NO and increased degradation of NO by reaction with superoxide. In this study, we found that the aortic eNOS protein attenuated in HFD-induced hyperlipidaemic mice. Eugenosedin-A and atorvastatin prevented HFD-induced eNOS protein reduction. Taken together, we suggest that both agents could inhibit HFD-induced hyperlipidaemia and inflammation.

From this study, it appeared that eugenosedin-A was comparable with atorvastatin in inhibiting hyperlipidaemia-induced inflammatory mediators. Therefore, we suggest that eugenosedin-A, as well as atorvastatin, could be

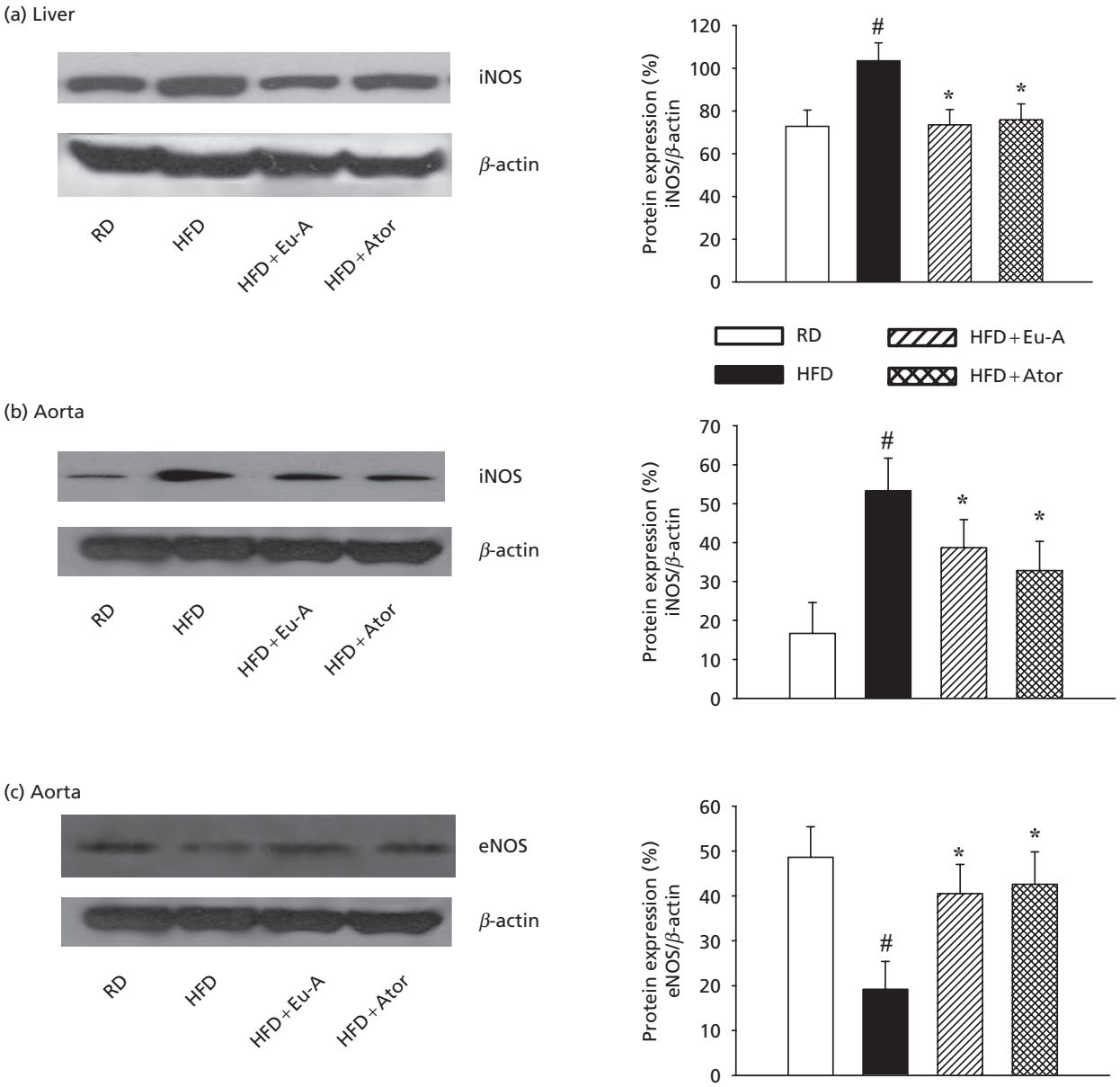

Figure 6 Effects of eugenosedin-A on inducible nitric oxide synthase protein expression.
Inducible nitric oxide synthase (iNOS) expression in liver (a) and aorta (b), and endothelial nitric oxide synthase (eNOS) protein expression in aorta (c) in high-fat diet (HFD) fed mice. Mice were fed either a regular diet (RD), a HFD, or a HFD with 5 mg/kg eugenosedin-A (Eu-A) or atorvastatin (Ator) for eight weeks. Each value represents the mean ± SE ($n = 8$). #$P < 0.05$ vs RD; *$P < 0.05$ vs HFD.

used in the control of obesity-related disorders. It will be necessary to investigate whether eugenosedin-A and atorvastatin inhibit cytokine-induced adhesion molecules. We have found that the inhibition of adhesion molecules by eugenosedin-A seems better than that of atorvastatin (unpublished data).

Conclusions

Eugenosedin-A and atorvastatin prevented HFD-induced inflammation and ameliorated eNOS downregulation that was closely related to the reduction of TNF-α and IFN-γ (proinflammatory mediators), p38, ERK and JNK (MAPKs pathway), p65 (Akt/IKKα pathway) and iNOS (Figure 7). We suggest that eugenosedin-A, like atorvastatin, could be developed in the control of HFD-induced metabolic syndrome.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

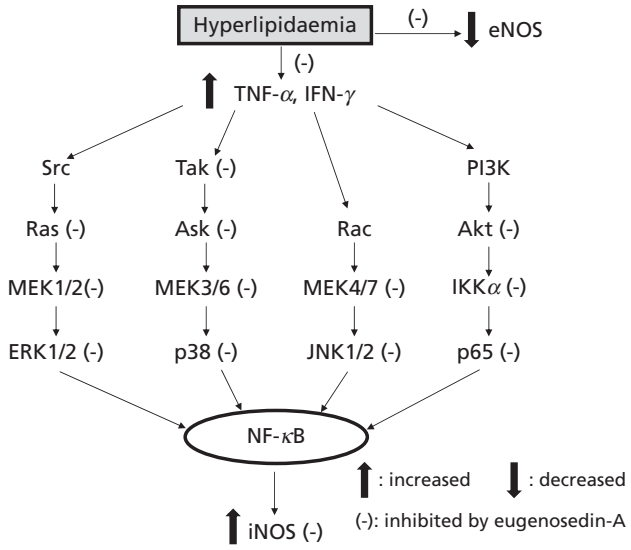

Figure 7 Hypothetical mechanisms of eugenosedin-A on hyperlipidaemia-induced inflammation.
Eugenosedin-A attenuated hyperlipidaemia-induced inducible nitric oxide synthase (iNOS) formation and endothelial nitric oxide synthase (eNOS) downregulation that were due to the reduction of tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (proinflammatory mediators), p38, ERK and JNK (MAPKs pathway), and p65 (Akt/IKKα pathway).

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