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Research Paper

Eugenosedin-A prevents hyperglycaemia, hyperlipidaemia and lipid peroxidation in C57BL/6J mice fed a high-fat diet

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Abstract

Objectives Eugenosedin-A is a serotonin (5-hydroxytryptamine; 5-HT) 5-$HT_{1B/2A}$ and $\alpha_1/\alpha_2/\beta_1$-adrenoceptor blocker with anti-oxidative, anti-inflammatory and free-radical scavenging activities. Previous reports demonstrated that 5-HT_{2A} blockers could diminish hyperlipidaemia. This study therefore aimed to investigate the possible uses and mechanisms of eugenosedin-A and other agents in treating hyperlipidaemia.

Methods C57BL/6J mice were randomly divided into seven groups, fed a regular diet or a high-fat diet alone or supplemented with one of five agents: eugenosedin-A, ketanserin, prazosin, propranolol or atorvastatin (5 mg/kg p.o.) for 8 weeks.

Key findings Compared with the regular diet, the mice fed the high-fat diet had significantly higher body weight and glucose, insulin and lipid levels. Brain malondialdehyde concentration was increased and liver glutathione peroxidase activity decreased. Addition of eugenosedin-A to the high-fat diet resulted in less weight gain and reduced hyperglycaemia, hyperinsulinaemia and hyperlipidaemia. Lipid and glucose homeostasis were related to decreased hepatic lipogenesis mRNAs and proteins (sterol regulatory element binding protein 1a, fatty acid synthase, sterol-CoA desaturase) and restored adipose peroxisome proliferator-activated receptor γ expression. Eugenosedin-A also enhanced low-density lipoprotein receptor mRNA expression.

Conclusions Eugenosedin-A may improve plasma lipid metabolism by increasing low-density lipoprotein receptor and peroxisome proliferator-activated receptor γ expression and diminishing sterol regulatory element binding protein 1a, fatty acid synthase and sterol-CoA desaturase. Reduction of plasma glucose and lipid levels may, in turn, reduce insulin concentration, which would explain the marked improvement in obesity-related hyperglycaemia and hyperlipidaemia. Furthermore, eugenosedin-A affected malondialdehyde concentration and glutathione peroxidase activity, suggesting it may have anti-peroxidation effects in mice fed a high-fat diet.

Keywords high-density lipoprotein (LDL) receptor; HMG-CoA reductase; hyperglycaemia; hyperlipidaemia; lipogenesis genes

Introduction

Diabetes mellitus has become a major world health issue. Modern high-fat diets (HFDs) are typically associated with large volume intake,[1] high energy intake and high weight gain. Excessive intake of fatty acids (FAs) tends to lead to an accumulation of tissue triglycerides, particularly in fat tissues. Subsequent increased lipolysis and increases in circulating FAs are associated with rising adipocytes and lipolysis and insulin resistance. This results in storage of FAs in non-adipose tissues such as muscle, pancreas and liver.[2] Diabetes mellitus develops along a continuum which appears to be triggered by insulin resistance from high fat intake together with increased circulation of FAs. The relationship between body weight and diabetes risk is therefore continuous and graded. Diabetes is associated with morbidity and mortality secondary to complications such as myocardial infarction, stroke and end-stage renal disease.[3]

5-Hydroxytryptamine (5-HT; serotonin) is a mitogen for vascular smooth muscle cells and is involved in arteriosclerosis.[4] Aviram and colleagues[5] demonstrated that 5-HT increases the uptake of low-density lipoprotein (LDL) via LDL receptors (LDLRs) and that of oxidised

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LDL via scavenger and other receptors in J-774A.1 murine macrophages. Serotonin-potentiated macrophage uptake of oxidised LDL was evaluated by increased lipoprotein degradation, which in turn resulted in enhanced cellular cholesterol ester accumulation. Suguro and colleagues[6] demonstrated that 5-HT interacts synergistically with oxidised LDL, its major components (lysophosphatidylcholine, 4-hydroxy-2-nonenal and reactive oxygen species), other vasoactive agents (thrombin, endothelin-1, angiotensin II and urotensin II) and monocyte chemoattractant protein-1 in inducing vascular smooth muscle cell proliferation.

The onset and progression of atherosclerosis is also exacerbated by hypertension, which can be well controlled using various antihypertensive agents (e.g. calcium antagonists, angiotensin-converting enzyme inhibitors, α_1-adrenoceptor blockers). Use of these agents also decreases the incidence of hypertension-related vascular complications such as apoplectic stroke. Antihypertensive agents also have various effects on blood lipid abnormalities and atherosclerosis. For instance, α_1-adrenoceptor blockers improve plasma lipid metabolism[7] and calcium antagonists have anti-atherosclerotic actions.[8] Because the presence of atherosclerosis is also closely correlated to abnormal blood lipid metabolism, an ideal antihypertensive agent would also improve plasma lipid metabolism. In hypercholesterolaemia, not only are there increased levels of cholesterol but also increased generation of free radicals, particularly in the vasculature and tissues, resulting in inflammatory disorders.[9] This may explain the anti-atherosclerotic effect of the antioxidant vitamin E, which can inhibit the oxidation of LDL oxidation as well as decrease O_2^- levels.[10]

Eugenosedin-A (4-{2-hydroxy-3-[1-(2-chlorophenyl-piperazinyl)]-propoxy}-3-methoxy-1-propylenyl- benzene), a derivative of isoeugenol, is a 5-$HT_{1B/2A}$ receptor and α_1/α_2/β_1-adrenoceptor blocker,[11] which is also capable of reducing inflammation and the scavenging of free radicals.[12] 5-HT_{1B} and 5-HT_{2A} receptors have been found to mediate 5-HT-evoked contractions in the large coronary artery in humans. Studies on vessels isolated from animal models of atherosclerosis have shown increased responses to serotonergic agents.[13] One previous report has also demonstrated that 5-HT_{2A} blockers could diminish hyperlipidaemia.[14] The role of eugenosedin-A in treating hyerlipidaemia could be related to its antagonism at 5-$HT_{1B/2A}$ receptors and α/β-adrenoceptors. The aim of this study was to determine the possible uses and mechanisms of eugenosedin-A and other agents (ketanserin, prazosin, propranolol and atorvastatin) in treating hyperlipidaemia. To date, not much research has been done on agents that have both serotonergic and α/β-adrenoceptor blocking activities in the treatment of hyperlipidaemia. In this study, we examined the mechanisms of action of eugenosedin-A on hyperlipidaemia, hyperglycaemia and lipid peroxidation in mice with HFD-induced obesity.

Materials and Methods

Drugs and chemicals

Ketanserin, prazosin, propranolol and atorvastatin were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Eugenosedin-A was synthesised in our laboratory. Brief, isoeugenol was reacted with epichlorohydrin, and the obtained intermediate epoxide was then reacted with *ortho*-chlorphenylpiperazine to yield eugenosedin-A. It was eluted by column chromatography and recrystallised with ethanol to achieve greater than 95% purity. Eugenosedin-A and other agents were solubilised in 5% absolute alcohol. Further dilutions were made in distilled water.

Animals

This study was approved by the Animal Care and Use Committee of Kaohsiung Medical University. Female mice (strain C57BL/6J), provided by the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan), were housed under constant environmental conditions of temperature and illumination (light between 7:30 and 19:30). Water, regular and high-fat diets were available *ad libitum*. After an acclimatisation period, the mice (6 weeks of age) were randomly divided into seven groups of eight rats: two control groups and five treatment groups, which were fed different diets for 8 weeks. One group of control mice was fed a regular diet ($n = 8$). The other groups were fed a HFD (TestDiet, Richmond, VA, US) containing 60 cal% fat, 21.4 cal% carbohydrates and 18.7 cal% protein. The five treatment groups had this diet supplemented with eugenosedin-A, ketanserin, prazosin, propranolol or atorvastatin (all 5 mg/kg) administered by oral gavage once daily at a regular time. Body weight was measured weekly. At the end of the study, the brain, liver, adipose tissue and serum were collected. The serum was used for biochemical assay. Tissues were stored in buffer solution at −80°C until analysis.

Measurement of serum biochemical parameters

Serum was collected by centrifugation. Serum glucose, triglyceride, cholesterol, LDL, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a Hitachi Clinical Analyzer 7070 (Hitachi High-Technologies Co. Tokyo, Japan). Insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Mercodia, Uppsala, Sweden).

Measurement of mRNA levels in liver and adipose tissue

Total RNA was extracted from liver and white adipose tissue by guanidine thiocyanate–water-saturated phenol/chloroform and then precipitated with acidic sodium acetate. The total RNA in the aqueous phase was precipitated with ice-cold isopropyl alcohol. Isolated RNA was detected by spectrophotometry (260 and 280 nm). One microgram of total RNA was reverse transcribed into cDNA using the Moloney murine leukaemia virus transcriptase and random hexamers as primers.

The six specific primers used were: peroxisome proliferator-activated receptor γ (PPAR-γ): sense, 5′-ACCACTCG-CATTCCTTTGGAC-3′, antisense, 5′-TCAGCGGGAAG-GACTTTAATG-3′; sterol regulatory element binding protein 1a: (SREBP1a) sense, 5′-GCGCTACCGGTCTTCTATCA-3′,

antisense, 5′-TGCTGCCAAAAGACAAGGG-3′; fatty acid synthase (FAS): sense, 5′-GATCCTGGAACGAGAACAC-3′, antisense, 5′-AGACTGTGGAACACGGTGGT-3′; sterol-CoA desaturase (SCD): sense, 5′-CGAGGGTTGGTTGTTGAT-CTGT-3′, antisense, 5′-ATAGCACTGTTGGCCCTGGA-3′; LDLR: sense, 5′-TCATCGCCCTGCTCCTTGCT-3′, antisense, 5′-GAGCCG TCAACACAGTCGACAT-3′; 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase: sense, 5′-CGGAATTCCGCCTGACATGCAGATTCTGGCAG-3′, antisense, 5′-GCTCTAGAGCTTGAACTCCCCACATTCTGTGC-3′; β-actin: sense, 5′-ACAGCTGAGAGGGAAATCGTG-3′, antisense, 5′-CTAGGAGCCAGGGCAGT-AATCT-3′.

Primers were added to 25 μl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM each dNTP, 5 μl cDNA, and 2.5 units Taq DNA polymerase to a final concentration of 0.5 M. The PCR conditions were as follows: denaturation (94°C, 1 min), annealing (57.5°C, 1 min) and extension (72°C, 1 min). RT-PCR products were electrophoresed in 1% agarose gels at 100 V and were stained with 0.5 μg/ml ethidium bromide. β-Actin was amplified as a control gene. The density of the PCR products was measured using a GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, US) The levels of mRNA were expressed as the ratio of signal intensity for each gene relative to that of β-actin.

Western blot analysis of protein expression in liver and adipose tissue

Tissue samples were obtained and frozen at −70°C before assay. Frozen tissues were homogenised on ice with a Polytron PT 3000 homogeniser (Kinematic AG, Littau, Switzerland) in a buffer composed of (mM): Tris-HCl 50, EDTA 0.1, EGTA 0.1, 2-mercaptoethanol 12 and phenylmethylsulfonyl fluoride 1 (pH 7.4). The homogenised tissues were centrifuged at 10 000*g* for 30 min and the supernatants were stored at −70°C until further analysis. Aliquots of tissue homogenates were used for protein assay (Bio-Rad) and Western blot analysis. Tissue homogenates containing 20 μg protein were reduced and separated on 7.5% SDS-PAGE gel using PhastSystem with PhastGel (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, US). 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 SREBP-1a, FAS, SCD, HMG-CoA reductase, LDLR and PPAR-γ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, US; 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 radiographic 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, Bethesda, MD, US) as described previously.[12]

Measurement of hepatic glutathione peroxidase and superoxide dismutase activities and brain malondialdehyde content

Liver samples were homogenised in a Polytron homogeniser in 50 mM HEPES buffer (pH 7.4) containing 5 mM $MgCl_2$, 1 mM EDTA, 100 mM KCl and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Glutathione peroxidase (GPx) activity was measured using a Bioxytech cGPx-340 assay kit (Oxis Research, Foster City, CA, US). Superoxide dismutase (SOD) activity was measured using a Bioxytech SOD-525 assay kit (Oxis Research).

Mice brains were dissected and homogenates prepared in 0.9% saline containing 10 mg/ml tissue. Samples were centrifuged at 600*g* and the resulting supernatant was used to estimate malondialdehyde (MDA) using an MDA-586 assay kit (Oxis Research).

Statistical analysis

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

Results

Body weight and weight gain

Control mice fed a regular diet gradually gained weight over the 8-week study period; those fed a HFD gained weight rapidly (Table 1). The HFD control group gained significantly more weight in total than the regular diet group (14.9 ± 1.8 g vs 7.3 ± 2.3 g). In the HFD group treated with the test agents, weight was gained but more gradually. Compared with the HFD controls, weight gain was inhibited by 45%, 40%, 42% and 44% in the groups treated with eugenosedin-A, ketanserin, prazosin and atorvastatin (5 mg/kg), respectively. However,

Table 1 Effects of eugenosedin-A and other agents on body weight and weight gain in mice fed a high-fat diet (HFD)

Group	Body weight (g)		Weight gain (%)
	Initial	Final	
Regular diet	17.6 ± 0.6	24.9 ± 2.4	29.3
HFD	18.2 ± 1.6	33.1 ± 2.6#	45.0#
HFD + eugenosedin-A	17.9 ± 1.7	26.1 ± 1.7*	31.4*
HFD + ketanserin	17.8 ± 1.3	26.8 ± 1.6*	33.6*
HFD + prazosin	18.3 ± 1.6	26.9 ± 1.8*	31.9*
HFD + propranolol	17.7 ± 1.4	30.3 ± 2.4	41.6
HFD + atorvastatin	18.2 ± 1.1	26.6 ± 2.5*	31.6*

Values are means ± SE (n = 8).
#$P < 0.05$ vs regular diet; *$P < 0.05$ vs HFD.

mice fed with lower doses of eugenosedin-A (1 and 3 mg/kg) did not show any significant effect on weight gain. No such inhibition was found in HFD-fed mice given propranolol (5 mg/kg).

Plasma glucose and insulin levels

Modest but significant hyperglycaemia developed in the HFD-fed control group compared with the mice fed the regular diet (Table 2). The HFD-fed control group had a much higher (3.7-fold) plasma insulin level than controls. Compared with the HFD-fed control group, the treatment groups had significantly lower plasma glucose levels. Insulin levels were significantly reduced in the groups treated with eugenosedin-A, ketanserin, prazosin and atorvastatin, but not in the group treated with propranolol (Table 2). Eugenosedin-A and three of the other agents markedly lowered plasma glucose and insulin levels in the groups fed the HFD. Although propranolol improved plasma glucose levels, it failed to reduce hyperinsulinaemia.

Plasma lipid levels

At the end of the study, the HFD-fed control group had a 1.4-fold higher plasma triglyceride level, 1.4-fold higher cholesterol level and a 1.8-fold higher LDL level than the regular-diet control group. Amongst the treatment groups, the eugenosedin-A-treated group had the greatest reduction in triglycerides (19%), cholesterol (14%) and LDL (30%). Hyperlipidaemia did not develop in the presence of ketanserin, prazosin or atorvastatin (Table 2). ALT and AST levels were not changed significantly.

Lipogenic genes in liver

To elucidate the molecular mechanism through which eugenosedin-A induces physiological changes, we used RT-PCR assays to probe for changes in the expression of genes involved in hepatic regulation of glucose homeostasis and lipid metabolism.

Comparing the mRNA expression of genes in the two control groups, the HFD-fed group had a significant increase in expression of SREBP1a mRNA and that of its target enzymes, FAS and SCD (Figure 1). Eugenosedin-A decreased the expression of SREBP1a mRNA, and subsequently FAS and SCD mRNAs. Prazosin and atorvastatin markedly reduced the expression of SREBP1a and FAS mRNAs. Ketanserin also clearly reduced SREBP1a and SCD, but not FAS, mRNAs. None of the agents reduced levels of HMG-CoA reductase mRNA. Interestingly, all of the agents except for propranolol significantly recovered levels of LDLR mRNA (Figure 2).

Table 2 Effects of eugenosedin-A and other agents on plasma biochemical parameters in mice fed a high-fat diet (HFD)

Group	Glucose (mg/dl)	Insulin (ng/ml)	Triglyceride (mg/dl)	TC (mg/dl)	LDL (mg/dl)	ALT (IU/l)	AST (IU/l)
Regular diet	105.6 ± 3.6	0.79 ± 0.14	118.3 ± 5.3	165.4 ± 8.1	70.3 ± 6.2	20.6 ± 1.5	37.2 ± 2.1
HFD	151.2 ± 4.2#	2.97 ± 0.36#	164.9 ± 5.8#	238.5 ± 10.6#	125.7 ± 5.8#	22.4 ± 1.4	39.6 ± 1.5
HFD + eugenosedin-A	109.4 ± 3.9*	0.87 ± 0.15*	134.1 ± 6.1*	204.3 ± 9.2*	87.9 ± 5.2*	24.2 ± 1.2	38.5 ± 1.5
HFD + ketanserin	118.7 ± 3.4*	0.92 ± 0.19*	139.0 ± 5.7*	207.7 ± 7.6*	80.7 ± 5.2*	23.1 ± 2.4	39.4 ± 1.4
HFD + prazosin	115.8 ± 4.1*	0.85 ± 0.14*	128.6 ± 6.9*	209.6 ± 9.4*	85.5 ± 6.8*	24.8 ± 1.6	40.6 ± 1.3
HFD + propranolol	121.6 ± 4.2*	2.63 ± 0.28	159.6 ± 7.4	232.8 ± 8.6	124.2 ± 6.3	22.5 ± 1.2	39.3 ± 1.6
HFD + atorvastatin	113.3 ± 3.8*	0.84 ± 0.13*	122.4 ± 7.9*	189.5 ± 8.8*	71.5 ± 7.2*	23.1 ± 2.3	40.9 ± 1.8

Values are means ± SE (n = 8).
#$P < 0.05$ vs regular diet; *$P < 0.05$ vs HFD.
TC, total cholesterol; LDL, low-density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

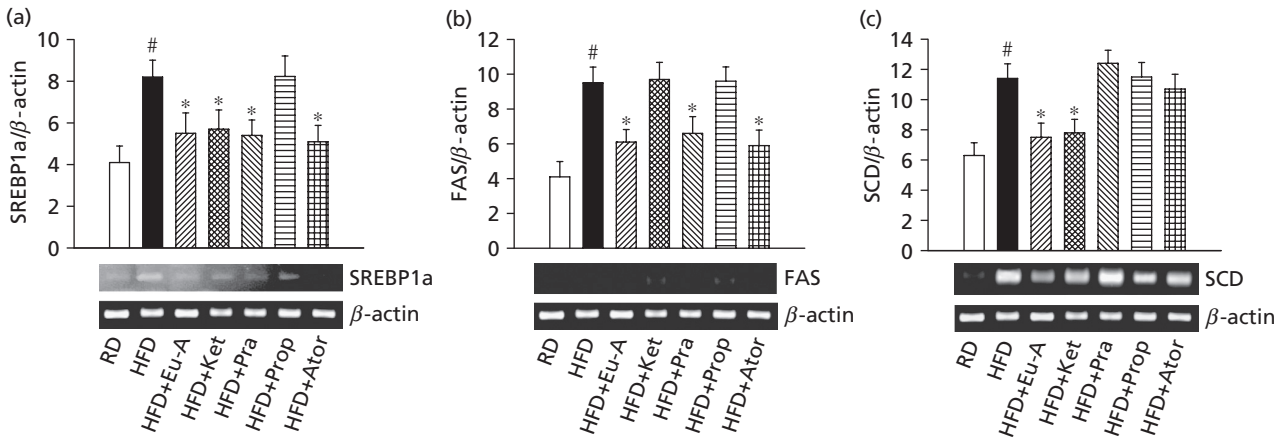

Figure 1 Effects of eugenosedin-A on (a) sterol regulatory element binding protein 1a (SREBP1a), (b) fatty acid synthase (FAS) and (c) sterol-CoA desaturase (SCD) mRNA expression in livers of mice fed a high-fat diet (HFD). Mice were fed a regular diet (RD) or HFD, alone or with 5 mg/kg eugenosedin-A (Eu-A), ketanserin (Ket), prazosin (Pra), propranolol (Prop) or atorvastatin (Ator) for 8 weeks. Values are means ± SE (n = 8). #$P < 0.05$ vs RD; *$P < 0.05$ vs HFD controls.

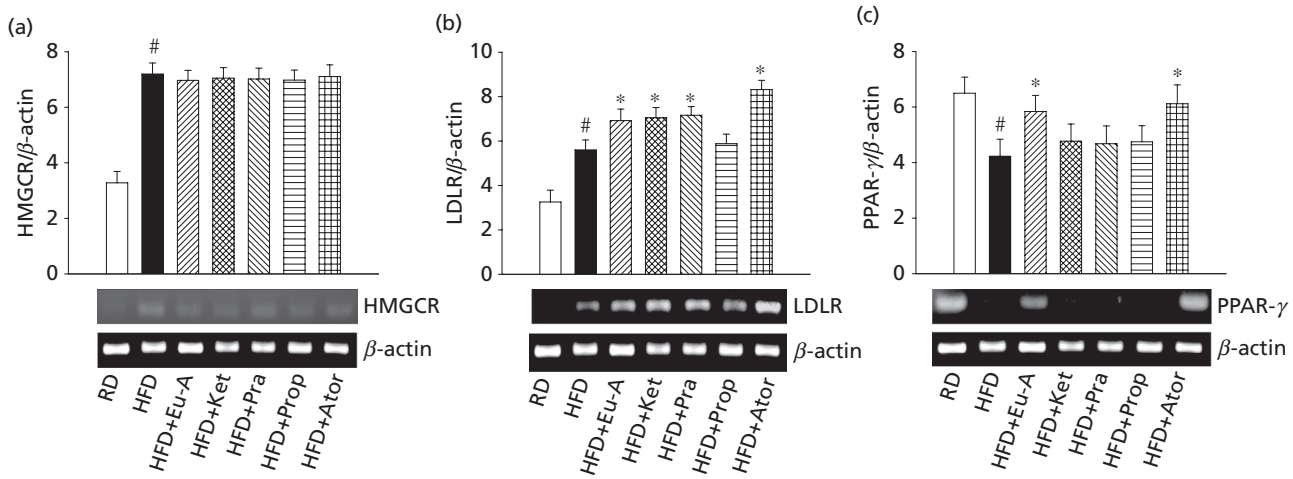

Figure 2 Effects of eugenosedin-A on (a) 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), (b) low-density lipoprotein receptors (LDLR) and (c) peroxisome proliferator-activated receptor γ (PPAR-γ) mRNA expression in livers of mice fed a high-fat diet (HFD). Mice were fed a regular diet (RD) or HFD, alone or with 5 mg/kg eugenosedin-A (Eu-A), ketanserin (Ket), prazosin (Pra), propranolol (Prop) or atorvastatin (Ator) for 8 weeks. Values are means ± SE (n = 8). $^{\#}P < 0.05$ vs RD; $^{*}P < 0.05$ vs HFD controls.

PPAR-γ expression in adipose tissue

PPAR-γ regulates glucose and lipid metabolism in adipose tissue. We performed a quantitative analysis of PPAR-γ using RT-PCR. The mRNA level of PPAR-γ in the HFD group was obviously lower than in the regular-diet group (Figure 2). Expression was significantly enhanced in the eugenosedin-A treated group. Atorvastatin also improved PPAR-γ mRNA levels whereas ketanserin, prazosin and propranolol did not.

Lipogenic and PPAR-γ protein expressions in liver and adipose tissue

SREBP1a, FAS and SCD protein levels were significantly increased in the HFD group. HMG-CoA reductase and LDLR protein levels were increased by eugenosedin-A in the HFD group and treated groups (Figure 3). Only eugenosedin-A and atorvastatin could recover PPAR-γ protein levels in the HFD-fed mice (Figure 4).

Hepatic glutathione peroxidase and superoxide dismutase activities and brain malondialdehyde content

Comparing the two control groups, the HFD-fed group had significantly lower GPx activity and enhanced MDA concentration (Table 3) but there was no significant difference between the groups in SOD activity. Eugenosedin-A, ketnaserin and atorvastatin were found to recover GPx activity in the HFD-fed mice. GPx activity was increased in the eugenosedin-A treated group (1216.9 ± 33.5 mU/ml vs 856.1 ± 29.3 mU/ml; $P < 0.05$). Upregulation of MDA

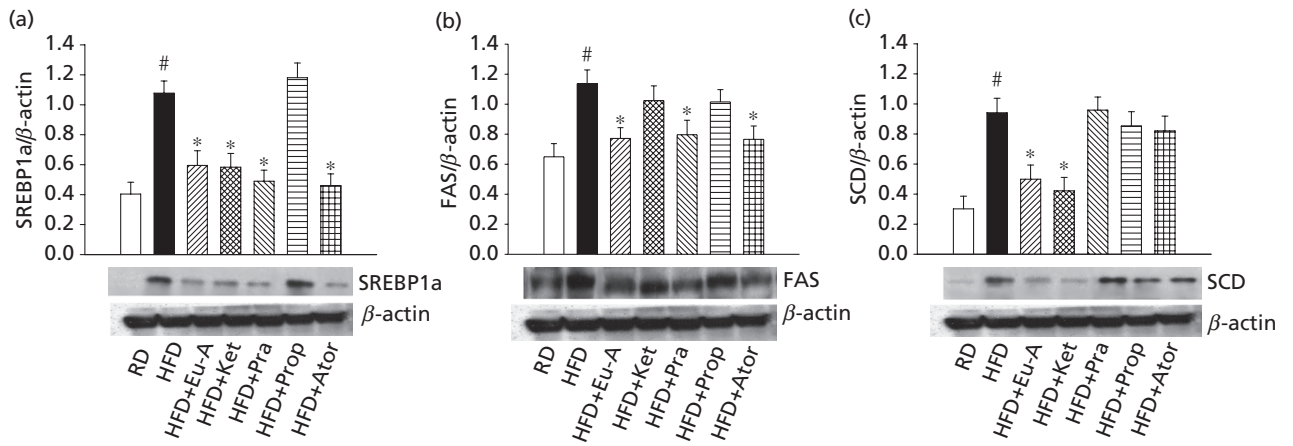

Figure 3 Effects of eugenosedin-A on (a) sterol regulatory element binding protein 1a (SREBP1a), (b) fatty acid synthase (FAS) and (c) sterol-CoA desaturase (SCD) protein expression in livers of mice fed a high-fat diet (HFD). Mice were fed a regular diet (RD) or HFD, alone or with 5 mg/kg eugenosedin-A (Eu-A), ketanserin (Ket), prazosin (Pra), propranolol (Prop) or atorvastatin (Ator) for 8 weeks. Values are means ± SE (n = 8). $^{\#}P < 0.05$ vs RD; $^{*}P < 0.05$ vs HFD controls.

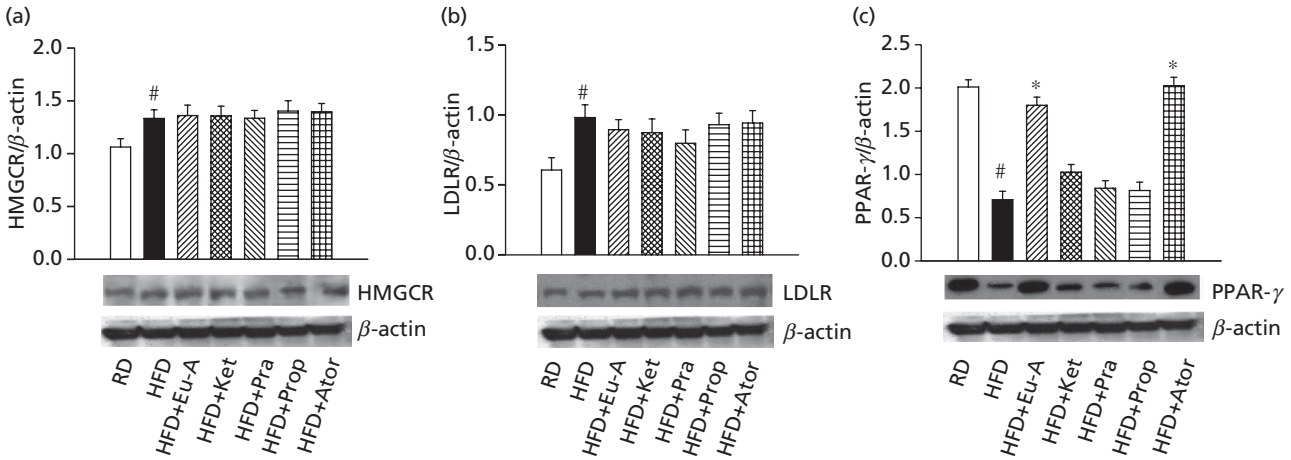

Figure 4 Effects of eugenosedin-A on (a) 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), (b) low-density lipoprotein receptors (LDLR) and (c) peroxisome proliferator-activated receptor γ (PPAR-γ) protein expression in livers of mice fed a high-fat diet (HFD). Mice were fed a regular diet (RD) or HFD, alone or with 5 mg/kg eugenosedin-A (Eu-A), ketanserin (Ket), prazosin (Pra), propranolol (Prop) or atorvastatin (Ator) for 8 weeks. Values are means ± SE (n = 8). $^{\#}P < 0.05$ vs RD; $^{*}P < 0.05$ vs HFD controls.

Table 3 Effects of eugenosedin-A and other agents on hepatic glutathione peroxidase (GPx) activity and brain malondialdehyde (MDA) content in mice fed a high-fat diet (HFD)

Group	Liver GPx activity (mU/ml)	Brain MDA (μM)
Regular diet	1205.8 ± 21.4	0.63 ± 0.08
HFD	856.1 ± 29.3#	1.12 ± 0.14#
HFD + eugenosedin-A	1216.9 ± 33.5*	0.75 ± 0.11*
HFD + ketanserin	1113.7 ± 30.1*	0.78 ± 0.08*
HFD + prazosin	844.5 ± 27.5	0.96 ± 0.07
HFD + propranolol	817.8 ± 31.6	0.91 ± 0.12
HFD + atorvastatin	1224.9 ± 21.3*	0.71 ± 0.11*

Values are means ± SE (n = 8).
$^{\#}P < 0.05$ vs regular diet; $^{*}P < 0.05$ vs HFD.

content was diminished by eugenosedin-A, ketanserin and atorvastatin but not by prazosin or propranolol.

Discussion

It has previously been reported that eugenosedin-A has both adrenergic and serotonergic activity, as well as an inhibitory effect on lipid peroxidation.[11,15] In this study, we investigated the mechanism behind its ability to lower both plasma glucose and lipids and its inhibitory effect on the synthesis of fatty acids in obese mice. To do this, we first made mice (C57BL/6J) obese by feeding them a HFD. Obese mice have pre-diabetic hyperglycaemia, insulin resistance and hyperlipidaemia.[16] We compared the effect of eugenosedin-A and four other agents (ketanserin, prazosin, propranolol and atorvastatin) on several parameters representative of the pre-diabetic state in these mice. Eugenosedin-A improved plasma glucose and lipid homeostasis significantly. Euegenosedin-A, ketanserin, prazosin and atorvastatin prevented obesity, hyperglycaemia, hyperinsulinaemia and hyperlipidaemia in the HFD-fed mice. Propranolol did not have these effects in obese mice, other than reducing hyperglycaemia.

Eugenosedin-A, ketanserin, prazosin and atorvastatin obviously prevented weight gain by suppressing the accumulation of glucose and lipids, whereas propranolol did not affect insulin and lipid concentrations in the same way (Tables 1 and 2).

High fat intake might lead to hyperglycaemia and hyperinsulinaemia. Therefore, most drug treatments of hyperlipidaemia-induced glucose metabolism disorders focus on enhancing insulin sensitivity and improving insulin secretion.[17] The neurotransmitter serotonin is an important regulator of energy balance, which in turn leads to glucose homeostasis. However, serotonergic pathways may also directly affect glucose homeostasis through the regulation of autonomic efferents and/or action on peripheral tissues. Several serotonergic compounds have been evaluated for clinical use in the treatment of obesity and type 2 diabetes.[18] Nonogaki and colleagues (2006) have reported that systemic administration of sarpogrelate, a 5-HT_{2A} antagonist, suppresses overfeeding, body weight gain and hyperglycaemia in obese Ay mice.[19] Ueshiba and Miyachi have demonstrated that doxazosin, an α_1-adrenoceptor antagonist, improves insulin resistance.[20] Blockade of postsynaptic α_2-adrenoceptors in the pancreatic beta-cell membrane produces subsequent hypoglycaemic effects.[21] β-Blockade-mediated suppression of glucagon release would have an additive inhibitory effect on hepatic glucose production.[22] First- and second-generation β-adrenoceptor antagonists are associated with worsening insulin resistance, deteriorating glycaemic control and severe hypoglycaemia.[23] Statins have also been found to ameliorate hyperglycaemia and insulin resistance.[24] In this study, we found that propranolol decreased HFD-induced hyperglycaemia, but not hyperinsulinaemia, though its reduction of glucose levels was inferior to that with the other agents we studied (Table 2). Eugenosedin-A acts differently from general β-blockers in that it also blocks 5-HT and α_1/α_2-adrenoceptors,[11] which may, through its

ability to ameliorate insulin resistance and insulin secretion, lead to the improvement of HFD-induced hyperglycaemia and hyperinsulinaemia.

In the present study, high fat intake led to increases in cholesterol, LDL and triglyceride concentrations (Table 2). Loschiavo and colleagues have reported that blood pressure is lowered, cholesterol and triglycerides levels reduced, and HDL increased in subjects taking ketanserin.[25] Doxazosin has been found to bring about changes in lipids characterised by significant reductions in total cholesterol, triglycerides and LDL, but no effect on HDL.[26] According to one study, β-adrenoceptor antagonists may have an additive effect on serum lipids.[27] Several clinical studies have found that HMG-CoA reductase inhibitors help reduce hyperlipidaemia. These agents are also known to inhibit intracellular cholesterol biosynthesis and induce the upregulation of LDLR. Statins may also influence the hepatic production and secretion of LDL and very-low-density lipoprotein by reducing cholesterol synthesis and diminishing the accumulation of lipoproteins.[28] In the current study, we found that eugenosedin-A, ketanserin, prazosin and atorvastatin, but not propranolol, reduced HFD-induced hyperlipidaemia (Table 2).

Free FAs and triglycerides have been reported to induce hyperinsulinaemia and insulin resistance. Suppression of the expression of enzymes involved in FA acid synthesis (SREBP1a, FAS) was observed to improve hyperinsulinaemia and insulin resistance.[29] To further explore the mechanisms behind the ability of eugenosedin-A and other agents to reduce hyperlipidaemia, we examined the mRNA expression of hepatic genes engaged in lipogenesis and lipolysis. SREBP1 activates the transcription of FAS and many other genes that are involved in lipid homeostasis.[30] FAS is a significant lipogenic enzyme that participates in energy metabolism;[31] SCD is the rate-limiting enzyme necessary for the biosynthesis of monounsaturated fatty acids.[32] We found that eugenosedin-A suppressed SREBP1a, FAS and SCD, thereby improving HFD-induced hyperlipidaemia (Figures 1 and 3).

HMG-CoA reductase is a rate-limiting enzyme in cholesterol biosynthesis; changes in its activity might affect LDLR activity. LDLRs in the liver play an important role in plasma lipoprotein metabolism. Cholesterol homeostasis is maintained by coordinated regulation of endogenous synthesis and exogenous uptake of lipoprotein cholesterol by LDLRs. When cholesterol is in homeostasis, the direct inhibition of HMG-CoA reductase can increase LDLR activity.[33] Total cholesterol and LDL have been found to be decreased by ketanserin,[34] which, as both a 5-HT_2 serotonergic and α_1-adrenoceptor antagonist, it achieves by suppressing HMG-CoA reductase activity.[35] Doxazosin has also been reported to inhibit cholesterol synthesis by reducing HMG-CoA reductase activity.[36] In our study, HMG-CoA reductase expression was increased in the HFD control and treated group (Figures 2 and 4). Ness and colleagues showed that atorvastatin caused a large compensatory induction of hepatic HMG-CoA reductase immunoreactive protein levels;[37] we found that eugenosedin-A had a similar effect.

Ness and colleagues also showed that atorvastatin increased hepatic LDLR mRNA but nor LDLR protein levels in a rat model.[38] This increase did not lead to an increase in cell surface LDLR protein levels, suggesting post-transcriptional regulation of the receptor. Furthermore, they showed an increased rate of LDLR protein degradation with statins, suggesting that statins might increase the rate of removal of LDL-cholesterol from blood by increasing the rate of receptor cycling. An α-adrenoceptor antagonist, or a combination of the α-adrenergic and 5-HT_{2A} serotonergic receptor antagonist, is thought to induce up-regulation of LDLR activity.[36] In monocyte-derived macrophages, serotonin has been reported to affect LDLR and scavenger receptor activity. Serotonin and/or serotonergic receptor antagonists may affect LDLR activity in fibroblasts.[39] We found that eugenosedin-A, ketanserin, prazosin and atorvastatin increased LDLR mRNA but not LDLR protein expression (Figures 2 and 4). This is an important mechanism through which eugenosedin-A reduces plasma cholesterol, suggesting that it may be able to ameliorate LDLR expression.

PPARs are members of the nuclear receptor superfamily. PPAR-γ has been identified as a key regulator of adipocyte differentiation and lipid metabolism, and its role in regulating glucose homeostasis has been established.[40] An increase in the expression of PPAR-γ increases the ability of insulin to mediate tissue glucose uptake and maintains glucose homeostasis.[3] As shown in Figures 2 and 4, we found that eugenosedin-A increased expression of PPAR-γ in adipose tissue. Atorvastatin, but not ketanserin, prazosin or propranolol, also increased the expression of PPAR-γ. We hypothesised that the activation of PPAR-γ by eugenosedin-A and atorvastatin may improve hyperinsulinaemia and hyperlipidaemia, as it lowers plasma glucose and lipid levels.

Glutathione (GSH), vital in the elimination of peroxidised materials and prevention of oxidative stress, is considered to be an anti-atherogenic enzyme.[41] MDA is considered a biomarker of lipid peroxidation. In hypercholesterolaemia, there is an increase in MDA levels and a decrease in GSH levels.[42] Statins have been reported to increase GSH,[43,44] and significantly reduce MDA levels in hyperlipidaemia.[43] In our study, hepatic GPx activity was reduced and brain MDA level was significantly increased in the HFD control group. In this study, eugenosedin-A, ketanserin and atorvastatin clearly ameliorated GPx activity and reduced MDA concentration. In our previous study, we found that eugenosedin-A was able to scavenge free radicals and inhibited lipid peroxidation.[15] Moreover, as suggested by Capela and colleagues,[45] we found that ketanserin was capable of improving GPx activity, and was also capable of reducing lipid peroxidation. Although previous studies have reported that prazosin and propranolol can inhibit lipid peroxidation,[46,47] we found that neither prazosin nor propranolol affected GPx activity or MDA levels.

Conclusions

In the present study, we found eugenosedin-A inhibited the development of hyperglycaemia, hyperlipidaemia and lipid peroxidation. In our in-vivo study, we treated hyperlipidaemic mice with eugenosedin-A for 8 weeks, and found a significant decrease in triglyceride and total cholesterol levels. Furthermore, eugenosedin-A was found able to activate PPAR-γ, resulting in significant reductions in glucose and lipid levels. We also found that ketanserin (5-HT_{2A} antagonist) and

prazosin (α_1-adrenoceptor antagonist) had similar effects to eugenosedin-A. Taken together, eugenosedin-A not only reduced weight gain in HFD-fed mice, but also corrected obesity-associated hyperglycaemia, hyperinsulinaemia, hyperlipidaemia and lipid peroxidation. Eugenosedin-A had a beneficial effect on glucose and lipid homeostasis, which it achieved through its ability to decrease hepatic lipogenesis genes and increase expression of PPAR-γ. In the future, we plan to investigate whether these agents are capable of ameliorating HFD-induced inflammation.

The current study provides the first evidence that eugenosedin-A is able to reduce glucose and lipid levels and thus may be useful in the future to improve obesity-related hyperglycaemia, hyperinsulinaemia and hyperlipidaemia and to scavenge free radicals, which would result in the reduction of obesity-induced lipid peroxidation.

Declarations

Conflict of interest

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

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