Effect of β_3 -adrenoceptor Activation on interaction of Renal Adrenoceptors and Angiotensin II Receptors in Aging ApoE$^{-/-}$ mice

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Abstract

To investigate the effect of β_3-adrenoceptor (β_3-AR) activation on the interaction of renal adrenoceptor (AR) and angiotensin II receptor (ATR) subtypes in aging apolipoprotein E knockout ($apoE^{-/-}$) mice. 10 C57BL/6J mice served as normal control group (group A) and 50 age-matched $apoE^{-/-}$ mice randomly divided into hyperlipidaemia model group (group B), atorvastatin treatment group (group C), low-dose β_3-AR agonist group (group D), high-dose β_3-AR agonist group (group E) and β_3-AR antagonist group (group F) were fed with high fat diet for 38 weeks, treatment in each group was given during the last 12 weeks of the high-fat diet. Then serum non-high density lipoprotein cholesterol (nHDL-C), blood glucose and insulin levels in each group were measured using an automatic biochemical detector. Expression of α_{1A}-AR, α_{1B}-AR, α_{2A}-AR, β_1-AR, β_2-AR, β_3-AR and angiotensin II type 1 (AT_1R) and type 2 (AT_2R) receptors in renal tissues were detected by RT-PCR and Western blot, respectively. Results showed that the serum nHDL-C, blood glucose and insulin levels significantly increased in hyperlipidaemia model mice ($P<0.01$), and the expression of α_{1A}-AR, AT_1R and AT_2R in renal tissues were significantly upregulated ($P<0.01$), with the expression of α_{1B}-AR, α_{2A}-AR, β_1-AR, β_2-AR and β_3-AR downregulated ($P<0.01$). Compared with group B, nHDL-C, glucose and insulin in aged $apoE^{-/-}$ mice decreased in group D and E ($P<0.01$). β_3-AR agonist treatment could down-regulate the expression of α_{1A}-AR and AT_1R ($P<0.05$), and increased α_{1B}-AR, α_{2A}-AR, β_1-AR, β_3-AR and AT_2R expression in group D and E ($P<0.05$), and thus resulted in improved lipid and glucose metabolism in aging hyperlipidaemia $ApoE^{-/-}$ mice, indicating that there are interaction between β_3- AR and different subtypes of AR, and between β_3-AR and ATRs, which may play a protective role in renal function injury caused by hyperlipidemia.

Background

It is well established that kidney plays an important role in maintaining the balance of blood pressure, water and electrolyte, the physical function of which is modulated by sympathetic nervous system (SNS) and rennin-angiotensin system (RAS) [1, 2]. Researches have shown that SNS and RAS, which are over-activated in many hyperlipidemia animal models, lead to the renal injury mediated by dyslipidemia [3, 4]. Dyslipidemia can induce epithelial-interstitial transformation of renal tubular epithelial cells, leading to renal tubule interstitial fibrosis, which may also promote mesangial cell proliferation and increase extracellular matrix deposition, resulting in glomerulosclerosis [5]. Our previous research found that activation of β_3-adrenoceptor (β_3-AR) in SNS and RAS can improve lipid and glucose metabolism disorders, thus delaying the occurrence of cardiac atherosclerosis and pancreatic injury in patients with hyperlipidemia [6, 7]. Whether β_3-AR activation has a protective effect on renal injury induced by hyperlipidemia and its mechanism remains unclear. The present study aimed to know the effects of β_3-AR agonist and antagonist on the expression of renal adrenoceptors (ARs) and angiotensin II receptors (ATRs), with particular attention to the issue that whether β_3-AR protected the renal function in high fat condition through the interaction between β_3-AR and other ARs, and between β_3-AR and ATRs.

Methods

Ethics statement

All animals were barrier-housed according to the standards for laboratory animals established by the People's Republic of China (GB14925-2001), and the experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Capital Medical University (Permit Number: SCXK-2012-0001). To minimize animal suffering, the lowest number of animals required for a statistically valid result was used. All experiments were performed under sodium pentobarbital anesthesia.

Animals

Homozygous ApoE$^{-/-}$ mice and wild type C57BL/6J mice (male, 10 weeks old, 22.85± 0.45g) were purchased from Beijing Vital River Laboratory Animal Technology Co Ltd.. All mice were adaptively barrier-housed for one week in an air-conditioned room without specific pathogens, with a light-dark cycle of 12 h. Then ApoE$^{-/-}$mice were given a high-fat diet containing 21% (w/w) fat and 0.15% (w/w) cholesterol from 11-48 weeks of age, while C57BL/6J mice (groupA, n=10) were given a normal diet. Autoclaved food and water were provided ad libitum [8].

At 36 weeks of age, ApoE$^{-/-}$mice (n=50) were randomly divided into the following 5 groups (n=10 per group): hyperlipidaemia model (group B), positive control (group C), low-dose β_3-AR agonist (groupD), high-dose β_3-AR agonist (group E)and β_3-AR antagonist (group F). Mice in groupA and B received vehicle (0.9% saline, intraperitoneal injection, twice a week). Mice in group C received atorvastatin at a dose of 10 mg/kg by gavage once a day. Mice in groupD received the β_3-AR agonist BRL37344 at a dose of 1.65 μg/kg by intraperitoneal injection, twice a week. Mice in groupE received the β_3-AR agonist BRL37344 at a dose of 3.30 μg/kg by intraperitoneal injection, twice a week. Mice in groupF received the β_3-AR antagonist SR59230A at a dose of 50 μg/kg by intraperitoneal injection, twice a week. All mice were treated for 12 weeks.

Reagents and drugs

The β_3-AR agonist BRL37344 and the β_3-AR antagonist SR59230A were obtained from Sigma Chemical Co. (St Louis, MO, USA). Atorvastatin was purchased from Pfizer Inc. (Dalian, China). Trizol reagent was produced by Invitrogen Co. (Carlsbad, CA, USA). Reverse transcription-polymerase chain reaction (RT-PCR) kit A3500 was obtained from Promega Co. (Madison, WI). Random primers were obtained from Invitrogen Co. (Shanghai, China). SYBR Green Real-Time PCR Master Mix kit was purchased from Katara Bio. (Shiga, Japan). Antibodies against α_{1A}-AR, α_{1B}-AR, α_{2A}-AR, β_1-AR, β_2-AR and β_3-AR, agiotensin II type 1 receptor (AT_1R) and β-actin were produced by Abcam Co. (California, USA). Secondary antibody was obtained from Sigma Chemical Co. (St Louis, MO. USA).

Measurement of glucose and insulin

All mice were fasted for 10 hours at 48 weeks of age and anesthetized using 1% sodium pentobarbital by intraperitoneal injection, and then blood samples were collected by retro-orbital sinus puncture. nHDL-C, Glucose (Glu) and Insulin (Ins) were detected using a Beckman CX7 (Beckman Coulter, Fullerton, CA, USA).

Quantitative real-time PCR

Total RNA was extracted from renal tissues with TRIZOL Reagent, which was then reverse transcribed into double-stranded cDNA using RT-PCR kit based on the manufacturer's protocol. Quantitative real-time PCR (qRT-

PCR) used a SYBR Green Real-Time PCR Master Mix kit (Katara Bio., Shiga, Japan). The reaction was performed as follows: 40 cycles of denaturation at 94℃ for 30s, annealing at 60℃ for 30s, and extension at 72℃ for 30s. Details of gene-specific primers were described in Table 1. The housekeeping gene β-actin was used as control. Results were normalized against β-actin and expressed as fold change for each gene using the $2^{-\Delta\Delta CT}$ method [9].

Table 1 Sequences of the primers used in this study. The sizes of the PCR products were given in base-pairs (bp).

Genes	NCBI number	Forward primers (5'-3')	Reverse primers (5'-3')	Sizes (bp)
α_{1A}-AR	NM_013461	GCAGCGGAGTAAGCAGTG	AGCCAGCAGAGGACGAAG	124bp
	NM-007416	TCCAGGGAAAAGAAAGCAGCCAA	GGGTAGATGATGGGGTTGAGGCA	188bp
α_{1B}-AR	NM_007417	CAAGATCAACGACCAGAAGT	GTGCGACGCTTGGCGATCT	120bp
α_{2A}-AR	NM_007419	TGGCTTACTGGCTTGTCTTG	TTTCCACTCGGGTCCTTG	135bp
	NM_007420	GGACAACCTCATCCCTAA	AGAGTAGCCGTTCCCATA	169bp
β_1-AR	NM_013462	CAGTCCCTGCCTATGTTTG	TTCCTGGATTCCTGCTCT	165bp
β_2-AR	NM_177322	TGCTCAGAAACGGGGACAC	CTCTGAAGTAGCCCACCTGTTA	177bp
β_3-AR	NM_007429	GATGGAGGGAGCTCGGAACT	AATTTGGAGTTGCTGCAGTTCAA	143bp
AT_1R	NM_007393	TCCATCATGAAGTGTGACGT	GAGCAATGATCTTGATCTTCAT	112bp
AT_2R				
β-Actin				

Western blot

Protein expression of α_{1A}-AR, α_{1B}-AR, α_{2A}-AR, β_1-AR, β_2-AR, β_3-AR and AT_1R in renal tissues was detected by western blot (WB). Total protein was extracted with RIPA buffer (50 mM Tris/HCl pH7.4, 150 mM NaCl, 2mM EDTA, 1% NP-40, 0.1% SDS). The protein concentration of the kidney was quantified using the Bradford protein assay. The samples were boiled for 5 min followed by loading on a 10% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. After transfer, the membrane was blocked for 2 h at room temperature with 5% nonfat dry milk in Tris-buffered saline-Tween (TBST; 20 mM Tris, 500 mM NaCl, pH 7.5, 0.1% Tween 20). The membranes were then incubated with diluted primary antibodies against α_{1A}-AR (rabbit polyclonal, 1:250), α_{1B}-AR (rabbit polyclonal, 1:1000), α_{2A}-AR (rabbit polyclonal, 1:500), β_1-AR (rabbit polyclonal, 1:1000), β_2-AR (rabbit polyclonal, 1:1000), β_3-AR (rabbit polyclonal, 1:1000) AT_1R (rabbit polyclonal, 1:1000) in 5% nonfat dry milk in TBST for 2 h at room temperature. After washing three times with TBST, the membranes were incubated with a secondary antibody (goat anti-rabbit IgG-HRP, 1:5000) conjugated to horseradish peroxidase in 2.5% nonfat dry milk in TBST for 1 h at room temperature. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL). The WB results were quantified by measuring the relative intensity compared with the control using Quantity one 4.6 (Bio-Rad, California, USA)[10].

Statistical analysis

The data was analyzed with SPSS23.0, and GraphPad Prism 8.0 was used for plotting. All data was expressed as means ± SE.M. One-way ANOVA was used to determine the difference between groups. Post-hoc comparisons were performed using a Bonferroni t-test (equal variances) or Dunn's multiple comparison test (unequal variances). A value of $P<0.05$ was considered statistically significant.

Results

Effects of β_3-AR on serum nHDL-C, glucose and insulin levels

Serum parameters at time of sacrifice were shown in Table 2. Compared with group A, $ApoE^{-/-}$ mice fed with high fat diet (group B) exhibited severe hyperlipidaemia, hyperglycemia and hyperinsulinemia ($P<0.01$). β_3-AR agonist groups (1.65 ug/kg and 3.30 ug/kg) at the end of treatment period had lower values of nHDL-C, glucose and insulin ($P<0.01$) compared with group B. However, no significant change in glucose was observed between group C and group B.

Table 2 Effects of β_3-AR on plasma glucose and insulin levels.

	A	B	C	D	E	F
nHDL-C(mmol/L)	0.70±0.05	$19.41±0.40^{a}$	$10.32±0.45^{ab}$	$14.31±0.31^{abc}$	$12.78±0.55^{abcd}$	$18.43±0.32^{a}$
Glu mmol/L	7.55±0.83	$15.39±1.05^{a}$	$14.25±0.94^{a}$	$11.51±0.72^{abc}$	$10.87±0.78^{abc}$	$15.04±0.10^{a}$
Ins ng/ml	1.28±0.26	$7.26±0.34^{a}$	$6.26±0.54^{ab}$	$4.79±0.25^{abc}$	$4.35±0.27^{abc}$	$7.14±0.31^{a}$

Results are expressed as the means ± S.E.M. Glu: glucose, Ins: insulin, A: normal control group, B: hyperlipidaemia model, C: positive control, D: low-dose β_3-AR agonist, E: high-dose β_3-AR agonist, F: β_3-AR antagonist. $^{a}P<0.01$ versus group A. $^{b}P<0.01$ versus group B. $^{c}P<0.01$ versus group C. $^{d}P<0.01$ vs group D.

Effects of β_3-AR on mRNA expression of renal ARs and angiotensin II receptors (ATRs)

The mRNA expression of renal ARs and ATRs were analyzed by qRT-PCR. As shown in Fig.1, compared with group A, the mRNA expression of α_{1A}-AR, AT_1R and AT_2R were significantly up-regulated ($P<0.01$), and α_{1B}-AR, α_{2A}-AR, β_1-AR, β_2-AR and β_3-AR were significantly down-regulated ($P<0.01$) in $apoE^{-/-}$ mice (group B). Compared with group B, atorvastatin (group C) and β_3-AR agonist (group D and E) both decreased α_{1A}-AR and AT_1R mRNA expression ($P<0.05$), with α_{1B}-AR, α_{2A}-AR, β_1-AR and AT_2R mRNA expression increased ($P<0.05$) and group E had the most significant effect on mRNA expression of these ARs and ATRs in renal tissue. There was no difference in β_2-AR mRNA between group B, C, D, E and F ($P>0.05$). No difference in β_3-AR mRNA existed between group B and group C ($P>0.05$), but it was significantly increased in group D and E ($P<0.05$), with no difference between the two groups ($P>0.05$). Compared with group B, there was no significant change in mRNA expression of renal ARs and ATRs in group F ($P>0.05$).

Effects of β_3-AR on protein expression of renal ARs and ATRs

As shown in Fig. 2, compared with group A, protein expression of α_{1A}-AR and AT_1R in group B were significantly increased (P<0.01), with protein expression of α_{1B}-AR, α_{2A}-AR, β_1-AR, β_2-AR and β_3-AR significantly decreased (P<0.01). Compared with group B, atorvastatin (group C) and β_3-AR agonist (group D and E) both decreased α_{1A}-AR and AT_1R protein expression (P<0.05), with α_{1B}-AR, α_{2A}-AR and β_1-AR protein expression increased (P<0.05) and group E had the most significant effect on protein expression of these ARs and ATRs in renal tissue. There was no difference in β_2-AR protein between B, C, D, E and F groups (P>0.05). No difference in β_3-AR protein existed between group B and group C (P>0.05), but it was significantly increased in group D and E (P<0.05), with significant difference between the two groups (P<0.05). Compared with group B, there was no significant change in protein expression of renal ARs and ATRs in group F (P>0.05). So high-dose β_3-AR agonist was more effective than atorvastatin.

Values were means ± S.E.M. Results shown were representative of four independent experiments (n=10). A: normal control group, B: hyperlipidaemia model, C: positive control, D: low-dose β_3-AR agonist, E: high-dose β_3-AR agonist, F: β_3-AR antagonist. The y-axis represented the relative expression level of target gene mRNA in each group based on group A. ^{a}P <0.01 versus group A. ^{b}P <0.05 versus group B. ^{c}P <0.05 versus group C.

Values were means ± S.E.M. Results shown were representative of four independent experiments (n = 10). The y axis represents the ratio of relative protein expression level of the target gene to the internal reference protein (β-actin) in each group. ^{a}P <0.01 versus group A. ^{b}P <0.05 versus group B. ^{c}P <0.05 versus group C.

Discussion

SNS and RAS play a key role in the regulation of renal function via their major neurotransmitters, and ARs and ATRs are widely distributed in the renal tissue. Studies have confirmed that β_3-AR can improve the metabolic disorders of glucose and lipid, and regulate the hemodynamic state [8]. Immunohistochemical staining results show that β_3-AR is mainly located in endothelial cells of renal arterioles and glomerular capillaries [11]. Excitatory β_3-AR can induce the glomerular afferent arteriolar vasodilation mediated by nitric oxide synthase (NOS) activation and nitric oxide (NO) release.[12]. This study mainly explored the effect of β_3-AR activation on interaction of renal ARs and ATRs in aging $ApoE^{-/-}$ mice. The results confirmed that β_3-AR agonist treatment could down-regulate the expression of α_{1A}-AR and AT_1R ($P < 0.05$), and increased α_{1B}-AR, α_{2A}-AR, β_1-AR, β_3-AR and AT_2R expression in group D and E ($P < 0.05$), and thus resulted in improved lipid and glucose metabolism in aging hyperlipidaemia $ApoE^{-/-}$ mice, indicating that there were interaction between β_3- AR and different subtypes of AR, and between β_3-AR and ATRs, which might play a protective role in renal function injury caused by hyperlipidemia.

Results showed that long-term high-fat diet in apoE-/- mice resulted in significantly increased serum nHDL-C, glucose and insulin levels. β_3-AR agonist intervention significantly decreased the serum nHDL-C, glucose and insulin levels, indicating that β_3-AR agonist improved glucose and lipid metabolism disorders under high fat condition. In atorvastatin group, the level of serum nHDL-C significantly reduced, while glucose and insulin levels

were not changed obviously, demonstrating that atorvastatin had obvious lipid-regulating effect, but had little effect on glucose metabolism.

Renal α_1-AR plays an important role in the regulation of renal hemodynamic and tubular function. Among the subtypes of α_1-AR, α_{1A}-AR and α_{1B}-AR are the functional ones which mediate renal cortical vasoconstriction and renal tubular sodium and water reabsorption. Noradrenalin acts on α_2-AR mediated renal vasodilation and reduced sodium and water reabsorption by increasing NO production, which plays a protective effect on the kidney [13–15]. Our results showed that the expression of α_{1A}-AR was significantly increased while α_{1B}-AR and α_{2A}-AR were significantly decreased in the model mice kidney, which aggravated the renal injury. It might be related to the fact that dyslipidemia, hyperglycemia and hyperinsulinemia activated the SNS, resulting in receptor compensatory desensitization or down regulation. [16, 17]. Studies have also found that adrenergic nerves could down-regulate α_1B-AR in the presence of elevated blood glucose and insulin resistance, while in order to maintain the effective regulation of α_1-AR, the function of postsynaptic α_1A-AR was enhanced [[18]. In this study, long-term use of atorvastatin and β_3-AR agonist both decreased the expression of α_{1A}-AR, and increased the expression of α_{1B}-AR and α_{2A}-AR in aging ApoE$^{-/-}$ mice. Atorvastatin and β_3-AR agonist promoted the recovery of the α_{1A}-AR, α_{1B}-AR and α_{2A}-AR expression, which alleviated renal injury. In addition, high-dose β_3-AR agonist was more effective on the regulation of receptor expression, possibly because both of β_3-AR and α-ARs were G protein-coupled receptors. β_3-AR activation interacted with α-ARs to induce or inhibit the formation of receptor dimer or cellular internalization, thus α_{1A}-AR, α_{1B}-AR and α_{2A}-AR expression tended to normal, which further improved renal function [19].

It has been established that β_1-AR and β_2-AR play a leading role in regulating renal blood flow and promoting sodium and chlorine reabsorption, as well as increasing renin secretion [20–22]. In the current study, the expression of β_1-AR, β_2-AR and β_3-AR in the renal tissues of the high-fat model group were significantly down-regulated, which might be related to the fact that chronic high fat treatment could activate the local renal RAS, AT_1R activation induced β_1-AR phosphorylation and desensitization via a PLC/PKC/c-src/PI3K pathway, and hyperinsulinemia inhibited β_3-AR gene transcription [23–25]. However, the expression of β_1-AR and β_3-AR in aging hyperlipidaemia ApoE$^{-/-}$ mice were up-regulated by β_3-AR agonist, which suggested that there were interactions between β_1-AR and β_3-AR. β_3-AR agonist improved glucose and lipid metabolism disorders in ApoE$^{-/-}$ mice, inhibited AT_1R-Gq signaling pathway, thus enhanced the role of β_1-AR, while β_3-AR could resist the receptor down regulation induced by log-term agonist stimulation [26, 27]. In contrast with this study, Seifert et al [28] reported that, β_3-AR agonist could induce β_3-AR desensitization of HEK293 cells from human embryonic kidney, probably because of the agonist concentration and exposure time. Compared with the high-fat model group, β_2-AR expression showed a trend of up-regulation after atorvastatin and β_3-AR agonist intervention, but the difference was not statistically significant, probably because β_1-AR in the renal tissue played a leading role, while β_2-AR had lower baseline expression level.

With local renal RAS activation, angiotensin II stimulates AT_1R, which can contract renal vessels, reduce vascular compliance, increase sodium reabsorption in renal tubules, activate SNS, induce inflammation and regulate the expression of bioactive substances [29–31]. On the other hand, activation of AT_2R can antagonize AT_1R via activating phosphotyrosine phosphatase and inactivating mitogen-activated protein kinase, which results in

vasodilation, increased urine sodium excretion, inhibition of renin secretion and cell hypertrophy [32]. In our research, the expression of AT_1R and AT_2R in the high-fat model group was up-regulated, which was consistent with the results of Ma et al [33], namely, lipid load could up-regulate the expression of AT_1R and AT_2R in glomerulus and proximal tubule epithelial cells. After the intervention of atorvastatin and β_3-AR agonist, the expression of AT_1R was down-regulated, with the expression of AT_2R further up-regulated, meanwhile the regulation of ATR was most significant in the high-dose β_3-AR agonist group. The reason might be related to the fact that the effect of β_3-AR agonist and atorvastatin on lipid metabolism attenuated renal RAS activity and down-regulated AT_1R expression. In addition, β_3-AR agonists could further down-regulate AT_1R and up-regulate AT_2R expression via β_3-AR dependent nitric oxide synthase activation and nitric oxide release [34–37]. AT_2R could antagonize AT_1R via NO-cyclic guanosine monophosphate (cGMP) pathway, and further reduce the expression of AT_1R by reducing AT_1R transcription and forming heterodimer with AT_1R, so that the ratio of AT_1R to AT_2R tended to normal [38–40].

In summary, long-term excited β_3-AR can adjust expression of receptors associated with the renal function in aging hyperlipidaemia $ApoE^{-/-}$ mice, suggesting that there were interactions between β_3- AR and different subtypes of AR, and between β_3-AR and ATR, which might play a protective role in renal function injury caused by hyperlipidemia.

Abbreviations

$apoE^{-/-}$: Apolipoprotein E knockout; nHDL-C: non-high density lipoprotein cholesterol; Glu: Glucose; Ins: Insulin; bp: base-pairs; SDS-PAGE: sodium dodecylsulfate polyacrylamide gel; TBST: Tris-buffered saline-Tween; ECL: enhanced chemiluminescence; AR: adrenoceptors; ATR: agitensin II receptors; NOS :nitric oxide synthase; NO: nitric oxide; cGMP: cyclic guanosine monophosphate.

Declarations

Ethics approval and consent to participate

The experiments in this study have been approved by the Committee on the Ethics of Animal Experiments of Capital Medical University (Permit Number: SCXK-2012-0001).All animals were barrier-housed according to the standards for laboratory animals established by the People's Republic of China (GB14925-2001), and all procedures were carried in accordance with the ARRIVE guidelines. (https://arriveguidelines.org).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are not publicly available yet, due to privacy concerns. Data are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Yanfang Li and Junying Song conceived and designed the experiments. Wei Wang successfully completed the whole project and was a major contributor in writing the manuscript. Fengde Li assisted the first author in completing all experiments on this subject. Fengde Li, Zhili Jiang and Junying Song helped to analyze the data and provided technical support during the trial. All authors agree to be personally responsible for their contributions and to ensure the accuracy or completeness of any part of the relevant issues. All authors read and approved the manuscript.

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Figures

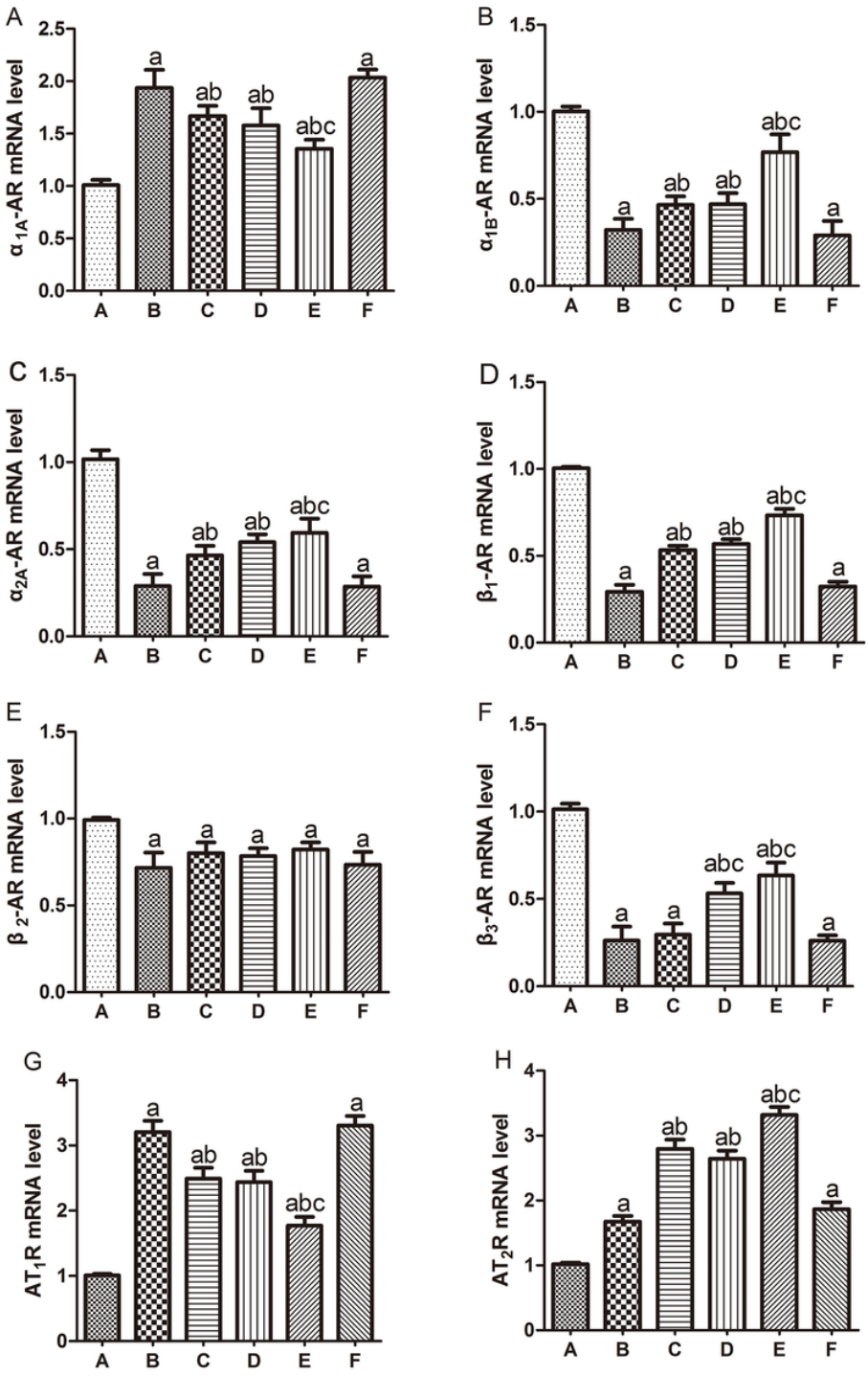

Figure 1

Effects of β_3-AR on the mRNA expression of adrenoceptors (ARs)

and agitensin II receptors (ATRs) in kidney.

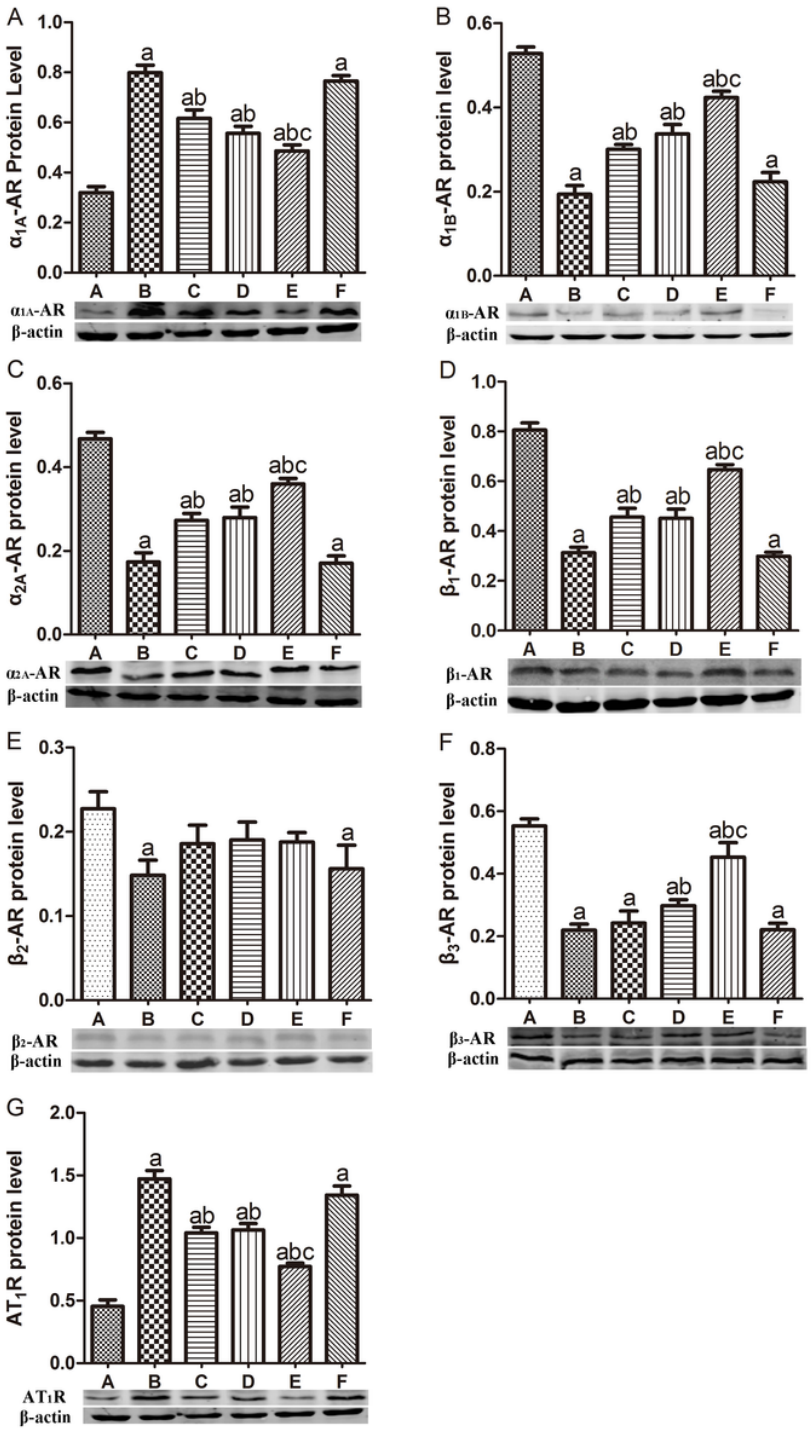

Figure 2

Effects of β_3-AR on the protein expression of adrenoceptors (AR) and agitensin II receptors (ATR) in kidney.