Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Adrenomedullin Attenuates Inflammation in White Adipose Tissue of Obese Rats Through Receptor-Mediated PKA Pathway

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Objective: Adrenomedullin (ADM) possesses therapeutic potential for inflammatory diseases. Consequently, the effects of ADM on inflammation in visceral white adipose tissue (vWAT) of obese rats or in adipocytes were explored in this study.
Methods: Male rats were fed a high-fat diet for 12 weeks to induce obesity, and obese rats were implanted with osmotic minipumps providing constant infusion of ADM (300 ng/kg per hour) and continued to be fed a high-fat diet for 4 weeks.
Results: When compared with the control group, endogenous protein expression of ADM and ADM receptors in vWAT and in lipopolysaccharide (LPS)-treated adipocytes was markedly increased. ADM significantly decreased the protein expression of the inflammatory mediators TNFα, IL-1β, cyclooxygenase-2, and inducible nitric oxide synthase in vWAT of obese rats and in adipocytes stimulated by LPS. It also inhibited the activation of the inflammatory signaling pathways MAPK and NF-κB induced by LPS in adipocytes. These effects of ADM in adipocytes were inhibited by the administration of ADM receptor antagonist and cAMP-dependent protein kinase (PKA) activation inhibitor.
Conclusions: ADM can inhibit inflammation in WAT in obesity, which may be mediated by the activation of ADM receptors and PKA.

Obesity (2021) **29**, 86-97.

Study Importance

What is already known?

- Low-grade chronic inflammation is associated with the adipose tissue dysfunction in obesity.
- Adrenomedullin (ADM) exerts a multitude of biological actions in both health and disease.
- ADM and its receptors' protein expressions in adipocytes and adipose tissue are found, and the plasma ADM level is elevated in children and adolescents with obesity.

What does this study add?

- ADM significantly decreased the protein expression of inflammatory mediators TNFα, IL-1β, cyclooxygenase-2, and inducible nitric oxide synthase in visceral white adipose tissue of obese rats and in adipocytes stimulated by LPS.
- ADM inhibited the activation of inflammatory signaling pathways MAPK and NF-κB induced by LPS in adipocytes.
- The anti-inflammatory effects of ADM in adipocytes were inhibited by the administration of ADM receptor antagonist and PKA activation inhibitor.

How might these results change the direction of research?

- ADM may be used for studying inflammation treatment in white adipose tissue in obesity.
- ADM may have effects on glucose and lipid metabolism in obesity.
- Potential protective effects of ADM on other complications of obesity, such as cardiovascular and kidney diseases, should be studied.

Introduction

Obesity is associated with the pathogenesis of some diseases such as diabetes, hypertension, and heart and kidney diseases (1-3). Many studies have conclusively demonstrated that inflammation plays a major role in obesity (4,5). Low-grade chronic inflammation is associated with the adipose tissue dysfunction in obesity (6). Abnormal production of proinflammatory cytokines in white adipose tissue (WAT) results in the development of metabolic disorders such as insulin resistance and atherosclerosis (6,7). Most individuals with obesity have elevated markers of inflammation, including the main proinflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin (IL)-1β, immune-associated cytotoxic factors such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and the activation of transcription factor of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways in adipocytes (8,9).

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Received: 7 March 2020; **Accepted:** 5 August 2020; **Published online 28 September 2020. doi:10.1002/oby.23012**

However, an excessive inflammatory response is an important pathogenic mediator for the development of obesity. Thus, suppression in the production of inflammatory-associated cytokines is important for the prevention and treatment of obesity-associated diseases (4).

Adrenomedullin (ADM), an endogenous 52 amino acid peptide, belongs to the calcitonin gene-related peptide family (10,11). It exerts a multitude of biological actions in both health and disease (10,11). ADM could serve as a novel therapeutic agent for the treatment of diseases mediated by ADM interaction with the seven-transmembrane domain calcitonin receptor-like receptor (CRLR) combined with a specific receptor activity-modifying protein (RAMP) (10,11). The ADM1 and ADM2 receptors consist of the CRLR combined with RAMP2 and RAMP3, respectively (10). The protein expression of the ADM peptide and ADM receptors have been detected in various tissues and organs, including blood vessels, skeletal muscles, heart, lungs, and nerve tissue (10). Moreover, their expression in adipocytes and adipose tissue also has been found (12,13). Plasma ADM levels are elevated in children with obesity, and the midregional ADM or proadrenomedullin plasma levels are high in adolescents with obesity, although the pathophysiological significance of ADM is largely unknown in obesity (14,15). Increasing evidence has indicated the potential benefits of ADM in anti-inflammatory properties. For instance, it has been shown to attenuate inflammation in retinopathy, sepsis, asthma, and chronic obstructive pulmonary disease (16-18). However, the anti-inflammatory effects of ADM in WAT in obesity, especially the underlying mechanisms, have not been studied.

In the present study, we determined whether ADM can attenuate inflammation in WAT of obese rats and inhibit lipopolysaccharide (LPS)-induced inflammation in adipocytes, and the possible mechanisms involved in the inhibitory responses were explored.

Methods

Experimental design

Experiment 1. The Cell Counting Kit-8 (CCK8) cell cytotoxicity test was used to determine the effect of ADM (10nM) and LPS (1 μg/mL) on cell growth for 24 hours in differentiated 3T3-L1 adipocytes.

Experiment 2. The ADM, C-reactive protein (CRP), and TNFα levels in plasma of obese rats were detected through the enzyme-linked immunosorbent assay (ELISA) method.

Experiment 3. The protein expression of ADM, CRLR, and RAMP2/3 was determined in visceral WAT (vWAT) of obese rats and in differentiated 3T3-L1 adipocytes under LPS (1 μg/mL) stimulation using the Western blot method.

Experiment 4. The effects of ADM on inflammation in vWAT from obese rats and LPS-induced inflammation in differentiated 3T3-L1 adipocytes were explored. ADM (10nM) was pretreated for 30 minutes, and then LPS (1 μg/mL) was added to the cell culture medium for 24 hours to observe the protein expression of the inflammation-related mediators TNFα, IL-1β, COX-2, and iNOS using the Western blot method.

Experiment 5. The effects of ADM (10nM) on the activation of the MAPK family (p38, extracellular signal-regulated protein kinase [ERK]1/2, and c-Jun NH2-terminal kinase [JNK]) and NF-κB were explored in differentiated 3T3-L1 adipocytes under LPS (1 μg/mL) stimulation by examination of the protein phosphorylation levels with the Western blot method.

Experiment 6. The mechanisms of ADM's action on LPS-induced inflammation and the activation of the inflammatory signaling pathways MAPK and NF-κB were investigated in differentiated 3T3-L1 adipocytes. The cAMP-dependent protein kinase A (PKA) inhibitor (P9115, 10^{-7} M) and ADM receptor antagonist (ADM22-52, 10^{-6} M) were used to explore whether ADM inhibits inflammation and the activation of inflammatory signaling pathways via the receptor-mediated PKA pathway.

Animals

Animal experiments were performed on male Sprague-Dawley rats (200-220 g). Rats were randomly divided into two groups. One group received a high-fat diet (45% of kilocalories as fat: 45% fat, 40% carbohydrate, 15% protein; Trophic Animal Feed Hightech Co. Ltd., Nantong, China) to induce obesity (OB), and the other group received a normal diet (12% of kilocalories as fat: 12% fat, 60% carbohydrate, 28% protein; Trophic Animal Feed Hightech) and served as control group for 12 or 16 weeks. They were housed in a temperature- and humidity-controlled room with a 12-hour light-dark cycle and were allowed access to rat chow and tap water ad libitum. The experiments complied with the Guidelines for the Care and Use of Laboratory Animals (NIH publication, 8th edition, 2011) and were approved by the Animal Experimental Ethics Committee of the Animal Core Facility of Nanjing Medical University (1601149-6, November 15, 2018). The criterion for the obese rats was that the body weight of the rats in the high-fat diet group was 20% more than that of the control group. The obese rats were further randomly divided into two groups ($n=8$/group), and they were fed a high-fat diet with saline or ADM (300 ng/kg per hour) treatment for 4 weeks. ADM was administered in saline through an Alzet Mini-osmotic Pump (Alzet model 2004, DURECT Corp., Cupertino, California) for infusion by subcutaneous implantation into the rats. After the end of the treatment, the visceral fat (inguinal, epididymal, mesenteric, and perirenal) was collected and weighed. Some of the visceral fat was fixed in 4% paraformaldehyde for immunofluorescent imaging. After 16 weeks of treatment, sodium pentobarbital was used to anesthetize rats by intraperitoneal injection. Plasma samples were obtained by centrifugation of heparinized blood for estimation of ADM, CRP, and TNFα levels. Finally, the plasma and visceral fat were kept at −80°C for further analysis.

Pump implantation

Aseptic surgical techniques were needed for this surgical implantation of ALZET pumps. Sodium pentobarbital (40 mg/kg) was used for anesthesia in rats. The site for pump implantation in rats was on the back, between and slightly posterior to the scapulae. A mid-scapular incision about 0.5 to 1.0 cm was made, and a hemostat was inserted into the incision for spreading the subcutaneous tissue caudally to create an appropriate pocket for the pump placement. After placement, the incision was closed with wound sutures. The wound and the health of the rats were monitored daily until completion of the study.

3T3-L1 cell line

Mouse 3T3-L1 preadipocytes (China Center for Type Culture Collection, Wuhan University, Wuhan, Hubei, China) were cultured in medium with DMEM, 10% fetal bovine serum (FBS), and 1% penicillin (100 U)/streptomycin (100 mg/mL) at 37°C in 5% CO_2 atmosphere.

After the confluence of the cells, differentiation was induced by 0.5mM 3-isobutyl-1-methylxanthine (IBMX), 1μM dexamethasone, 2μM rosiglitazone, and 10 μg/mL of insulin for 3 days, and then the fresh culture medium was used (10 μg/mL) every 3 days until the cells were fully differentiated into mature adipocytes. The differentiation process was 9 days. The differentiated mature adipocytes were used for experiments.

Cell viability assay

A CCK8 kit was used to detect the effects of ADM and LPS on cell viability. The differentiated 3T3-L1 adipocyte suspension (100 μL) was added to a 96-well plate and cultured for 24 hours at 37°C in a cell incubator. Different test substances (10 μL) were administrated into the plate. After 24 hours of incubation, the CCK solution (10 μL) was applied into each well and incubated for 3 hours. Finally, the absorbance at 450 nm was determined using a microplate reader (ELX800; BioTek, Winooski, Vermont).

Oil red O staining

The differentiated 3T3-L1 adipocytes in a six-well plate were identified using Oil red O staining. The cells were fixed with 4% formalin after washing with phosphate-buffered saline (PBS) and washed again with deionized water. Oil red O solution (0.6% Oil red O dye in isopropanol) was used to combine intracellular lipid. The stained lipid droplets were visualized using an inverted microscope as shown in Figure 1A.

Immunofluorescent imaging

The vWAT obtained from control and obese rats was fixed in 4% paraformaldehyde and then embedded in optimal cutting temperature (O.C.T.) compound (tissue freezing medium). Adipose tissue was obtained by frozen section methods, cut at 7-mm thickness, and mounted on gelatin-coated slides. The differentiated adipocytes were grown on poly-L-lysine pretreated coverslips with or without LPS treatment (1 μg/mL) for 24 hours. The coverslips were fixed with methanol and acetone mixture (1:1) followed by washing with PBS. The sections and coverslips were permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri), washed with PBS three times, and blocked with 10% donkey serum in PBS for 1 hour. The sections and coverslips were then incubated with the first primary antibody; rabbit ADM, CRLR, RAMP2, and RAMP3 polyclonal antibodies from Abcam Trading (Shanghai) Company Ltd (Pudong, Shanghai, China) were applied at 1:100 dilution

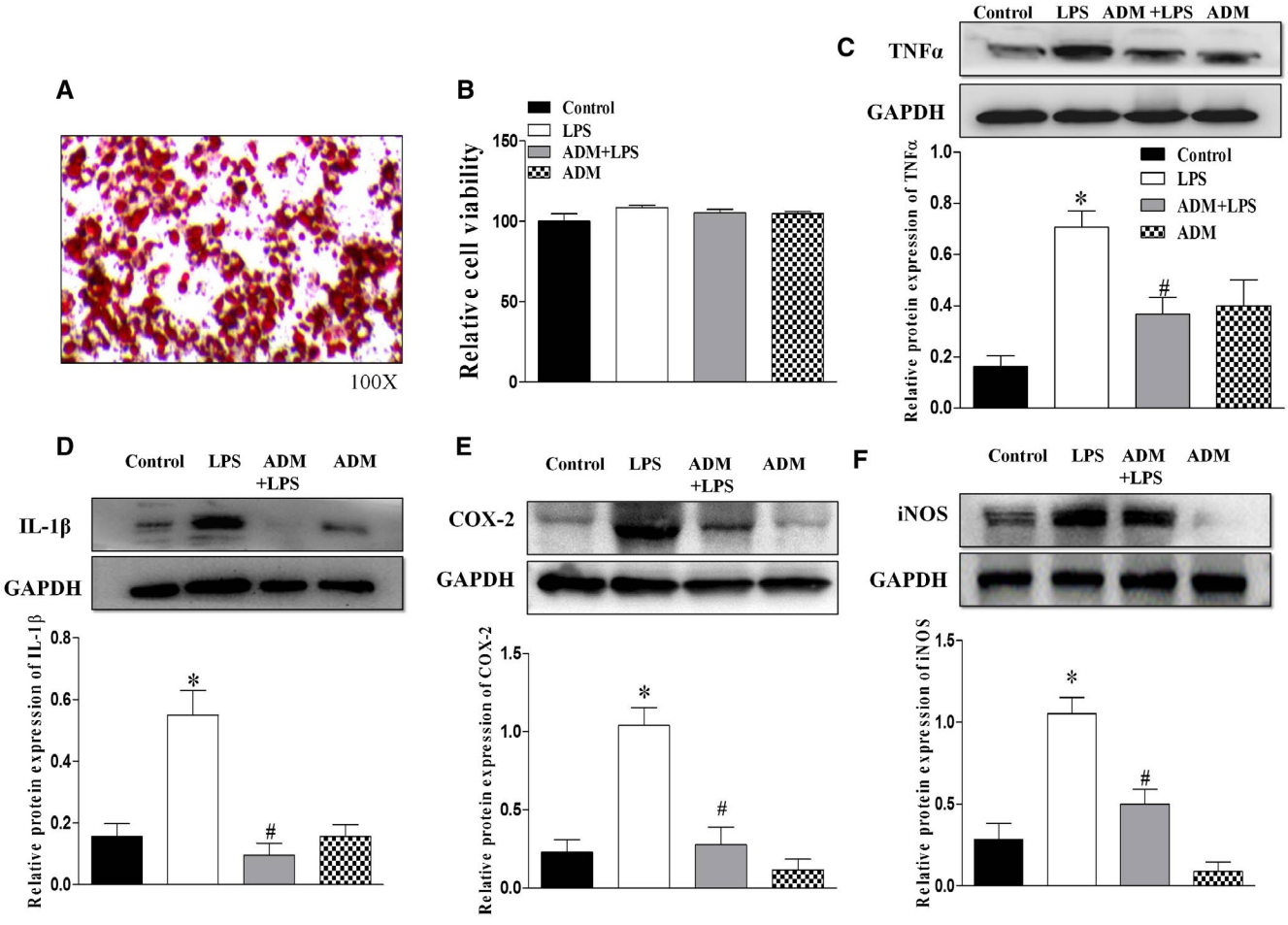

Figure 1 (**A**) Differentiated 3T3-L1 adipocytes in a six-well plate were identified using Oil red O staining. (**B**) ADM and LPS had no significant toxic effect on cell viability, and (**C-F**) ADM treatment for 24 hours reduced LPS-induced protein expression of TNFα, IL-1β, COX-2, and iNOS in the differentiated 3T3-L1 adipocytes. Cell lysates were analyzed by the Western blotting method. GAPDH was used as an internal control for Western blotting analysis. Each value indicates mean ± SEM; $n = 3$ to 5. $^{*}P < 0.05$ vs. control group. $^{\#}P < 0.05$ vs. LPS group. [Color figure can be viewed at wileyonlinelibrary.com]

overnight at 4°C, followed by three washes with PBS. Sections and coverslips were then incubated with polyclonal goat/anti-rabbit secondary antibody IgG H&L (Alexa Fluor 594, 1:200 dilutions) tetramethylrhodamine-isothyocyanate (Proteintech, Wuhan, China). Corresponding normal IgG from Santa Cruz Biotechnology (Shanghai) Co., Ltd. (Pudong New District, Shanghai, China) served as a negative control. Nuclei were stained with 4,6-diamino-2-phenyl indole from (Invitrogen, Pudong New District, Shanghai, China). The fluorescence images were captured by an Olympus BX51 fluorescence microscope.

ELISA

The levels of ADM, CRP, and TNFα in the plasma were measured by ELISA kits according to the manufacturer's instructions. The respective final solution was read by a microplate reader (ELX800; BioTek). The ELISA kits for ADM, CRP, and TNFα were purchased from Phoenix Pharmaceuticals (Burlingame, California), RayBiotech (Peachtree Corners, Georgia), and R&D systems (Minneapolis, Minnesota), respectively.

Western blotting

Briefly, equal amounts of protein extracts from adipocytes or vWAT were separated by polyacrylamide gel electrophoresis (PAGE) and then were electrotransferred to polyvinylidene difluoride membranes (19). Primary antibodies were used against ADM, CRLR, RAMP2, RAMP3, TNFα, IL-1β, iNOS, COX-2, phosphor-IκBα, IκBα, phosphor-NF-κB p65, NF-κB p65, phosphor-MAPK, nonphosphorylated MAPK including phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, or GAPDH overnight at 4°C. Horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG were used as secondary antibodies. The immunoblots were visualized with the electrochemiluminescence (ECL) Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA). Protein band intensities were normalized with nonphosphorylated MAPK, IκBα, total-NF-κB p65, or GAPDH levels. The signals were quantified using Odyssey Imaging System (LI-COR Biosciences, Lincoln, Nebraska).

Reagents and antibodies

ADM (molecular formula: $C_{242}H_{381}N_{77}O_{75}S_5$; sequence: H-Tyr-Arg-Gln-Ser-Met-Asn-Gln-Gly-Ser-Arg-Ser-Thr-Gly-Cys-Arg-Phe-Gly-Thr-Cys-Thr-Met-Gln-Lys-Leu-Ala-His-Gln-Ile-Tyr-Gln-Phe-Thr-Asp-Lys-Asp-Lys-Asp-Gly-Met-Ala-Pro-Arg-Asn-Lys-Ile-Ser-Pro-Gln-Gly-Tyr-NH_2) was purchased from Bachem (Bubendorff, Switzerland). IBMX, dexamethasone, rosiglitazone, and insulin were from

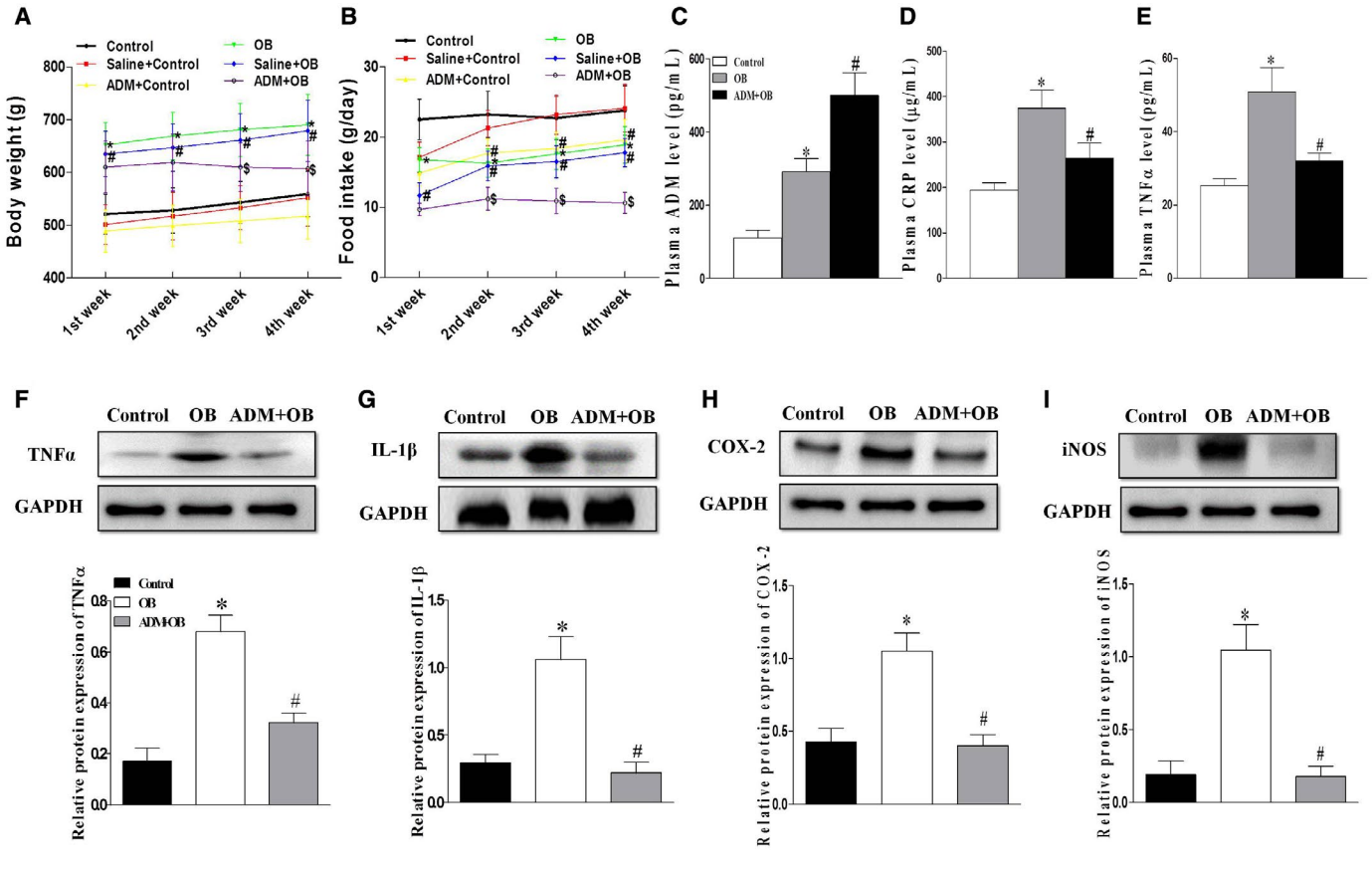

Figure 2 (**A,B**) Food intake and body weight parameters were measured in rats during chronic application of ADM. (**C,D**) Plasma levels of ADM, CRP, and TNFα were increased in obese rats, and (**F-I**) ADM chronic administration for 4 weeks not only decreased plasma TNFα and CRP levels but also inhibited protein expression of inflammatory mediators TNFα, iIL-1β, COX-2, and iNOS in visceral white adipose tissue of obese rats. Tissue lysates were analyzed by Western blotting method. GAPDH was used as an internal control for Western blotting analysis. Each value indicates mean ± SEM. (**A-E**) $n = 6$ to 8; (**F-I**) $n = 3$ to 5. $^{*}P < 0.05$ vs. control group. (**A,B**) $^{\#}P < 0.05$ vs. saline + control. (**B**) $^{\$}P < 0.05$ vs. saline + OB. (**C-I**) $^{\#}P < 0.05$ vs. OB group. [Color figure can be viewed at wileyonlinelibrary.com]

Sigma-Aldrich. LPS, PKA inhibitor P9115, and ADM receptor antagonist ADM22-52 were from Anaspec (Fremont, California). DMEM, FBS, 0.25% trypsin-EDTA, streptomycin/penicillin, and trypsin were obtained from Thermo Fisher Scientific (Pudong New District, Shanghai, China). The ADM, CRLR, RAMP2/3, and TNFα antibodies were from Abcam (Cambridge, UK). The IL-1β antibody was obtained from Proteintech (SANYING, Wuhan, China). The COX-2 antibody was from Cayman Chemical (AmyJet Scientific Inc., Hongshan, Wuhan, China). The iNOS antibody was purchased from BD Biosciences (Pudong New District, Shanghai, China). The phosphor-IκBα, total-IκBα, phosphor-NF-κB p65, total-NF-κB p65, phospho-p38, total-p38 MAPK, phospho-ERK1/2, total-ERK1/2, phospho-JNK, and total-JNK antibodies were obtained from Cell Signaling Technology (Shanghai) Biological Reagents, Inc. (Pudong New District, Shanghai, China).

Statistics

We analyzed the data using GraphPad Prism version 5.00 (San Diego, California). All data illustrated are expressed as mean (SEM). Differences in the mean values between two groups were assessed by unpaired *t* test. One-way ANOVA was used for data analysis of more than two groups followed by Bonferroni post hoc analysis. In all cases, $P<0.05$ was considered statistically significant.

Results

Physical and metabolic parameters and plasma CRP and TNFα levels in rats

Body weight and visceral fat weight were significantly increased in 16-week-old rats with high-fat diet feeding compared with age-matched control rats with normal diet. Plasma glucose, insulin, triglycerides, and total cholesterol were higher in OB rats than in control rats (Supporting Information Table S1). ADM treatment not only markedly decreased the body weight (Figure 2A) and visceral fat weight (Supporting information Table S1) but also significantly reduced the increased plasma triglycerides, insulin, CRP, and TNFα

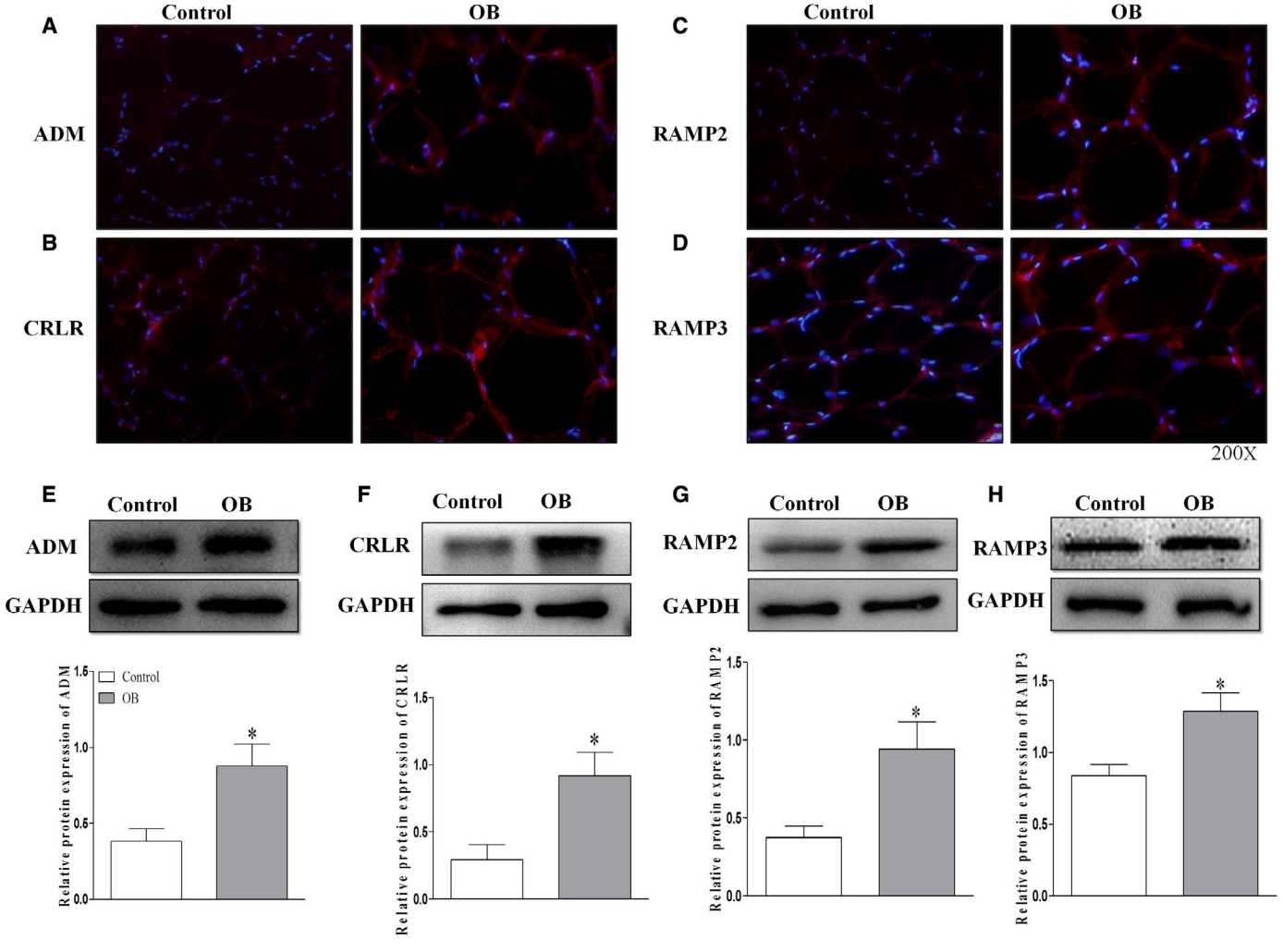

Figure 3 (**A-D**) Immunofluorescent staining and (**E-H**) Western blotting showed that the obese rats had increased endogenous protein expression of ADM, CRLR, RAMP2, and RAMP3 in visceral white adipose tissue. ADM, CRLR, RAMP2, and RAMP3 protein levels were normalized to GAPDH protein expression. Original magnification, 200×; $n=3$ to 4. Each value indicates mean ± SEM. *$P<0.05$ vs. control group. [Color figure can be viewed at wileyonlinelibrary.com]

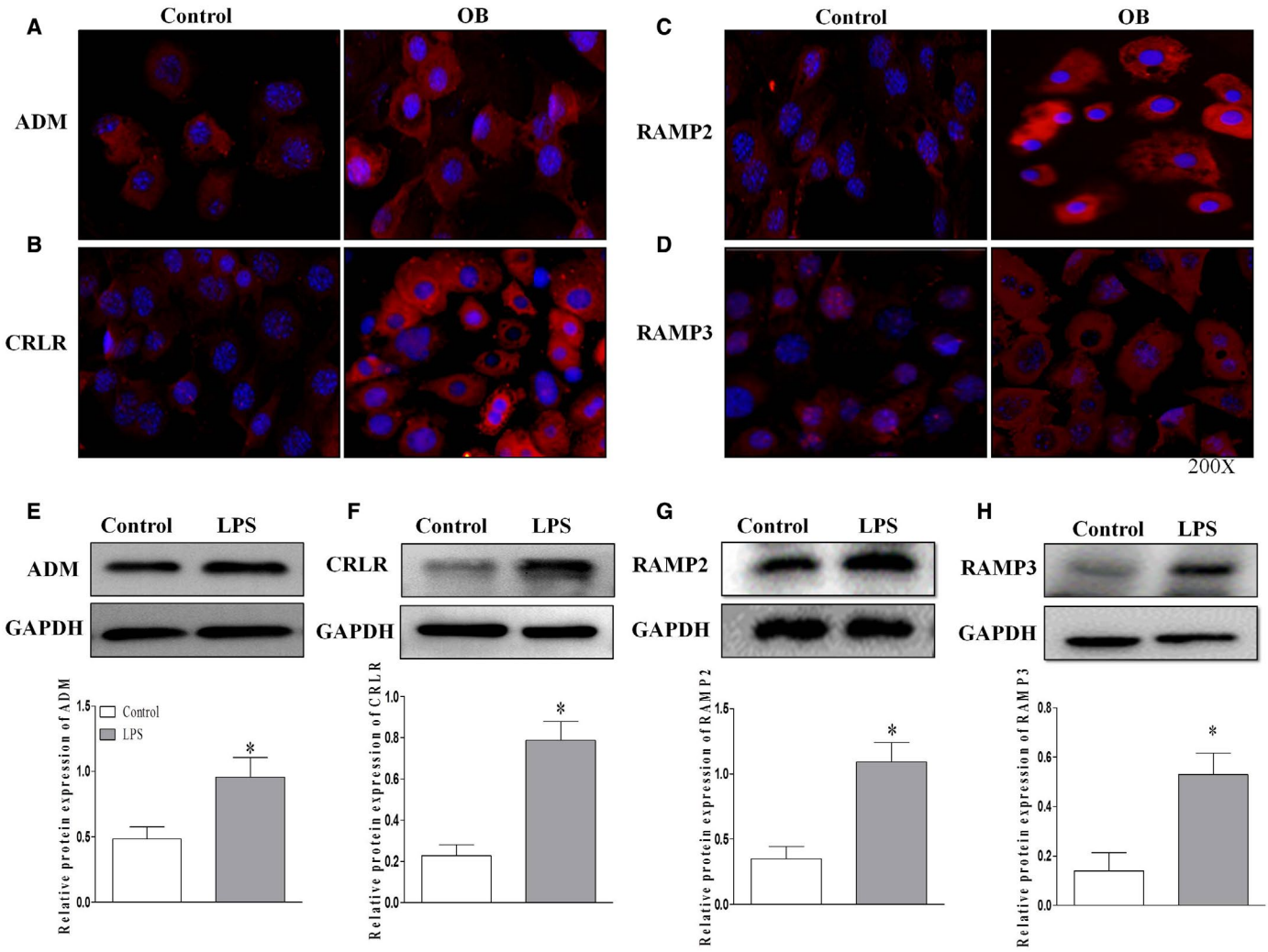

Figure 4 (**A-D**) Immunofluorescent staining and (**E-H**) Western blotting showed that LPS increased endogenous protein expression of ADM, CRLR, RAMP2, and RAMP3 in the differentiated 3T3-L1 adipocytes. ADM, CRLR, RAMP2, and RAMP3 protein levels were normalized to GAPDH protein expression. Original magnification 200×; $n = 3$ to 5. Each value indicates mean ± SEM. *$P < 0.05$ vs. control group. [Color figure can be viewed at wileyonlinelibrary.com]

levels (Supporting Information Table S1 and Figure 2D-2E). Although the concentration of glucose and total cholesterol levels in plasma did not significantly differ between OB and ADM-treated obese rats, the glucose level had a decreased tendency in ADM-treated obese rats (Supporting Information Table S1). Moreover, ADM treatment inhibited food intake in control and obese rats (Figure 2B). The effects of ADM on physical and metabolic parameters are worth further exploration.

Effects of ADM and LPS on cell viability in differentiated 3T3-L1 cells

To test whether chemicals applied in this study produced any cytotoxic effect, the differentiated 3T3-L1 adipocytes were treated with LPS (1 μg/mL), ADM (10nM), and LPS plus ADM for 24 hours. As shown in Figure 1B, LPS alone or ADM alone did not produce significant changes in cell viability measured with the CCK8 assay. Moreover, LPS plus ADM also failed to result in obvious injury in the differentiated 3T3-L1 adipocytes.

ADM protein levels in plasma and protein expression of ADM and receptor system CRLR, RAMP2, and RAMP3 in vWAT and adipocytes

ADM levels were higher in the plasma of rats with obesity induced by HFD compared with control rats with normal diet (Figure 2C), and ADM subcutaneous infusion by pump further increased the ADM levels in plasma in obese rats. Moreover, protein expression of ADM and ADM receptor system CRLR, RAMP2, and RAMP3, quantified by the Western blot method, was significantly higher in vWAT from obese rats compared with control rats (Figure 3E-3H), and it also was significantly increased in LPS-treated adipocytes (Figure 4E-4H). Furthermore, immunofluorescent staining indicated that protein expression was significantly increased in vWAT of OB rats (Figure 3A-3D)

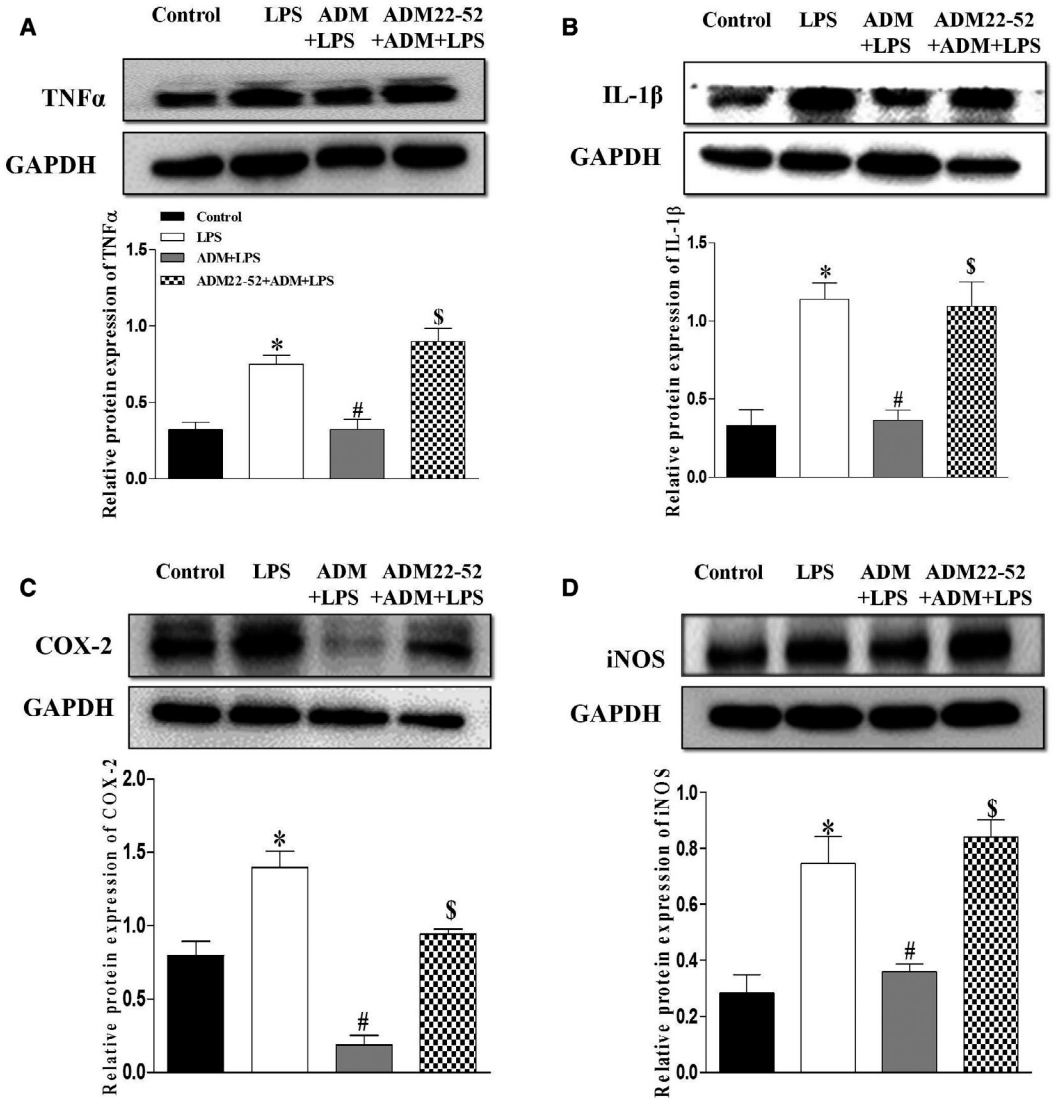

Figure 5 Blocking of ADM receptor with ADM22-52 (10^{-6} M) inhibited effects of ADM on LPS-induced protein expression of (**A**) TNFα, (**B**) IL-1β, (**C**) COX-2, and (**D**) iNOS in the differentiated 3T3-L1 adipocytes. Cells were pretreated for 30 minutes with ADM22-52. Following treatment with ADM for 24 hours, cell lysates were analyzed by the Western blot method. GAPDH was used as an internal control for Western blotting analysis. Each value indicates mean ± SEM; $n = 3$ to 5. $^{*}P < 0.05$ vs. control group. $^{\#}P < 0.05$ vs. LPS group. $^{\$}P < 0.05$ vs. ADM + LPS group. [Color figure can be viewed at wileyonlinelibrary.com]

and in LPS-stimulated adipocytes compared with the control group (Figure 4A-4D).

ADM administration suppresses inflammation in vWAT and in differentiated 3T3-L1 cells

We investigated a potential therapeutic effect of ADM on inflammation in vWAT of obese rats or in differentiated 3T3-L1 adipocytes stimulated by LPS. Obese rats were subcutaneously infused with saline (vehicle) or ADM (300 ng/kg per hour) by osmotic minipump for 4 weeks. The results showed that there were significant increases in protein expression of the inflammatory mediators TNFα, IL-1β, COX-2, and iNOS in vWAT of obese rats (Figure 2F-2I) or in differentiated adipocytes after LPS treatment for 24 hours (Figure 1C-1F), which was significantly reduced by ADM treatment. It indicated that ADM administration effectively ameliorated inflammation in WAT in obesity or in adipocytes.

Effects of ADM receptor antagonist and PKA activation inhibitor pretreatment on ADM response to LPS-induced inflammation

In many studies, the ADM receptor downstream signal is mostly transduced by the adenylyl cyclase–cAMP–PKA system (20,21). To study the mechanisms of ADM on LPS-induced inflammation, the differentiated 3T3-L1 adipocytes were pretreated with ADM receptor antagonist ADM22-52 (10^{-6} M) or PKA activation inhibitor P9115 (10^{-7} M) before ADM treatment. Our results showed that ADM22-52 (Figure 5A-5D)

or P9115 (Figure 6A-6D) significantly attenuated the inhibitory effects of ADM on LPS-induced protein expression levels of the inflammatory mediators TNFα, IL-1β, COX-2, and iNOS, which suggested that ADM via the activation of its receptor and PKA effectively inhibited inflammation.

ADM prevented LPS-induced inflammatory signal pathways, and ADM receptor antagonist and PKA activation inhibitor pretreatment inhibited ADM response to LPS-induced inflammatory signal pathways

To demonstrate an involvement of the signal transduction and mechanisms of the effects of ADM on LPS-induced inflammation in differentiated 3T3-L1 adipocytes, activation of MAPK and NF-κB was evaluated. The activation of the MAPK family (p38, ERK1/2, and JNK) and NF-κB is very important in regulation of cellular inflammatory responses (22,23). Moreover, the phosphorylation of IκBα and the subsequent translocation of the NF-κB p65 subunit is associated with NF-κB activation. In protein extracts from LPS-stimulated differentiated 3T3-L1 adipocytes, elevated levels of LPS-induced phosphorylation of p38, ERK1/2, and JNK were observed when compared with the control group without altering total p38, ERK1/2, and JNK levels, and LPS also significantly stimulated IκBα and NF-κB p65 phosphorylation in differentiated 3T3-L1 adipocytes. Interestingly, these increased phosphorylation protein levels were significantly reduced by ADM treatment. The differentiated 3T3-L1 adipocytes were pretreated with ADM receptor antagonist ADM22-52 (10^{-6} M, Figure 7A-7D) or PKA activation inhibitor P9115 (10^{-7} M, Figure 8A-8D)

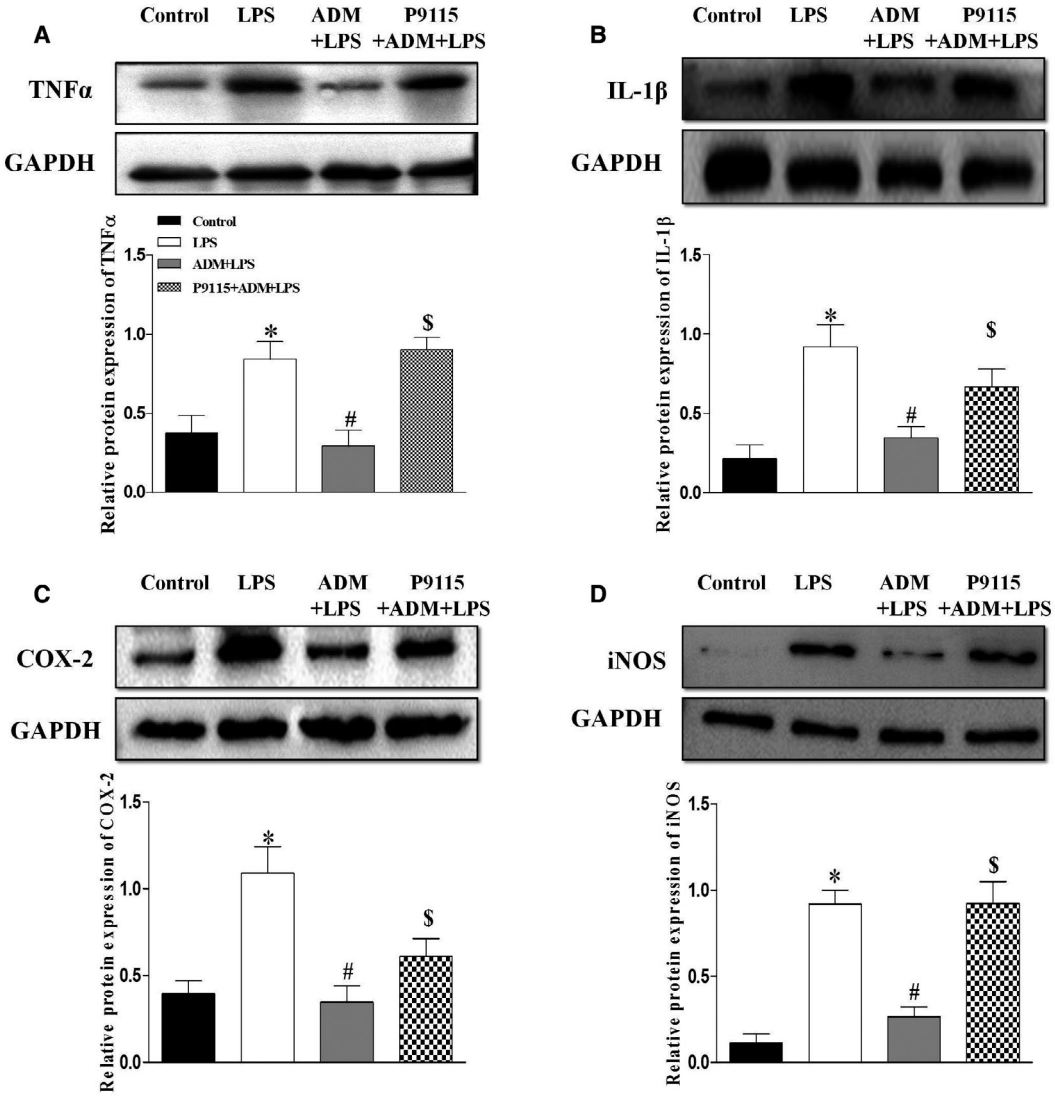

Figure 6 Inhibition of PKA activation with P9115 (10^{-7} M) suppressed effects of ADM on LPS-induced protein expression of (**A**) TNFα, (**B**) IL-1β, (**C**) COX-2, and (**D**) iNOS in the differentiated 3T3-L1 adipocytes. Cells were pretreated for 30 minutes with P9115. Following treatment with ADM for 24 hours, cell lysates were analyzed by the Western blot method. GAPDH was used as an internal control for Western blotting analysis. Each value indicates mean ± SEM; $n = 3$ to 5. $^{*}P < 0.05$ vs. control group. $^{\#}P < 0.05$ vs. LPS group. $^{\$}P < 0.05$ vs. ADM + LPS group.

before ADM treatment. The results showed that ADM22-52 or P9115 also effectively attenuated the inhibitory effects of ADM on LPS-induced activation of MAPK and NF-κB. These data confirm that ADM can prevent LPS-induced activation of inflammatory signaling pathways MAPK and NF-κB, and the inhibition of inflammatory signal pathways of ADM was regulated through the receptor-PKA activation.

Discussion

We have for the first time provided evidence that ADM effectively attenuated inflammation in WAT of obese rats. It also inhibited inflammation and the activation of inflammatory signal pathways of MAPK and NF-κB caused by LPS in the differentiated adipocytes, and these effects of ADM in the differentiated adipocytes were effectively attenuated by the inhibition of the ADM receptor and PKA activation. Moreover, our results showed that ADM and its receptor components, including CRLR, RAMP2, and RAMP3, were higher in WAT from obese rats compared with control rats, and the expression levels in differentiated adipocytes were enhanced under LPS stimulation *in vitro*.

High levels of circulating pro-ADM have been shown to predict clinical outcomes and survival (24,25). Increased pro-ADM levels found in obesity (14,15,26) may reflect disease severity, including cardiovascular or renal diseases as well as insulin resistance related to obesity. The increased ADM levels in obesity may suggest that ADM has a protective role against obesity complications. In this study, the levels of ADM in plasma or in WAT were higher in rats with obesity compared with controls. Furthermore, in the differentiated adipocytes under LPS stimulation, the ADM level was increased. Therefore, the high levels of ADM that we observed in obese rats may represent a compensatory mechanism against inflammation in WAT through the anti-inflammatory action of ADM.

Inflammatory cytokines in WAT can contribute to chronic inflammation and result in the development of obesity-related diseases (27-29). ADM, a biologically active peptide, has pleiotropic activities, including

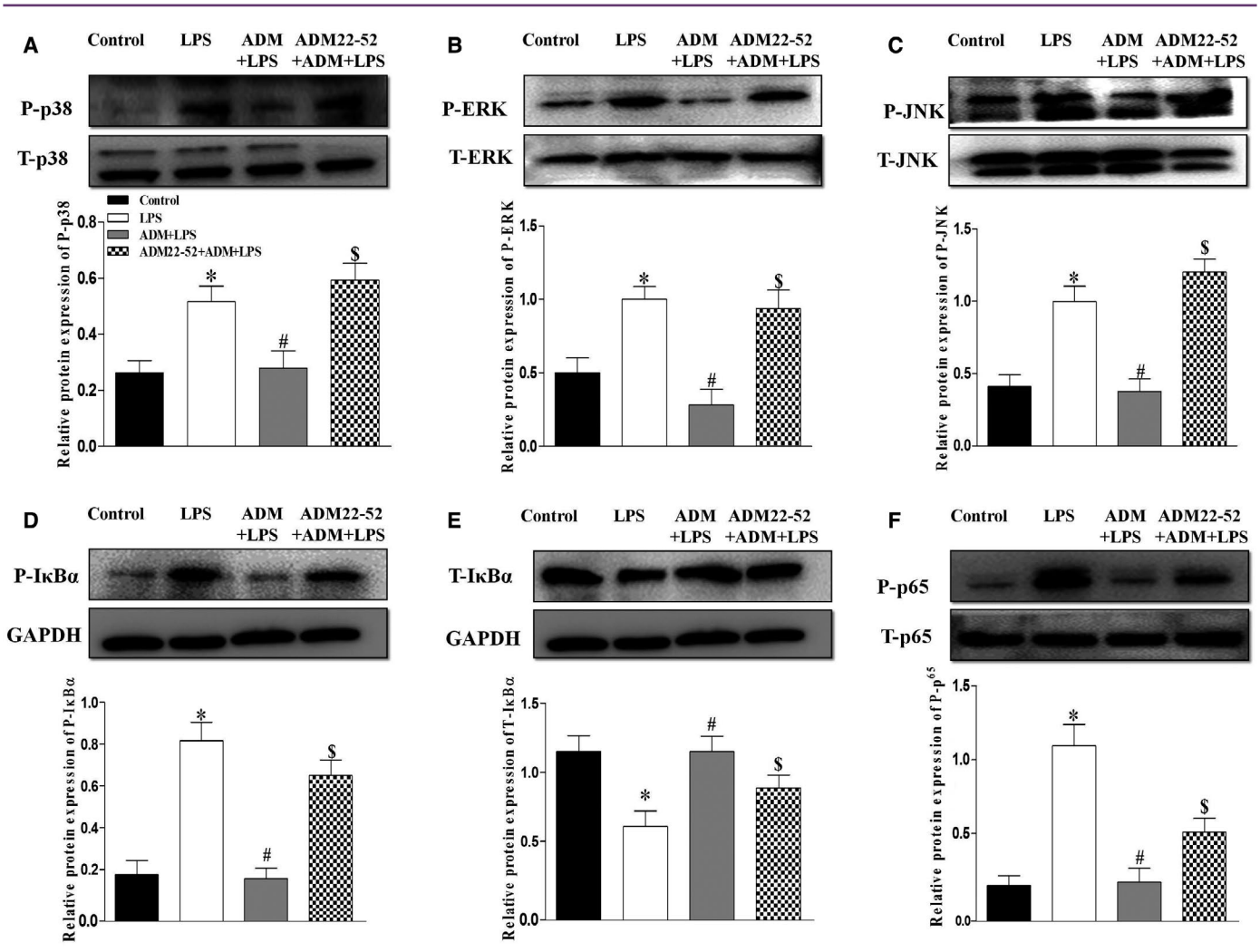

Figure 7 Blocking of ADM receptor with ADM22-52 (10^{-6} M) inhibited effects of ADM on LPS-induced phosphorylation of MAPK (P-p38, P-ERK1/2, and P-JNK) and NF-κB in the differentiated 3T3-L1 adipocytes. Cells were pretreated for 30 minutes with ADM22-52. Following treatment with ADM for 24 hours, each extract was prepared to determine the levels of (**A-C**) phosphorylated MAPK, (**D,E**) IκBα and total IκBα, and (**F**) p65 by the Western blot method. Total MAPK (p38, ERK1/2, and JNK), p65, and GAPDH were used as an internal control. Each value indicates mean ± SEM; $n = 3$ to 5. $^{*}P < 0.05$ vs. control group. $^{\#}P < 0.05$ vs. LPS group. $^{\$}P < 0.05$ vs. ADM + LPS group.

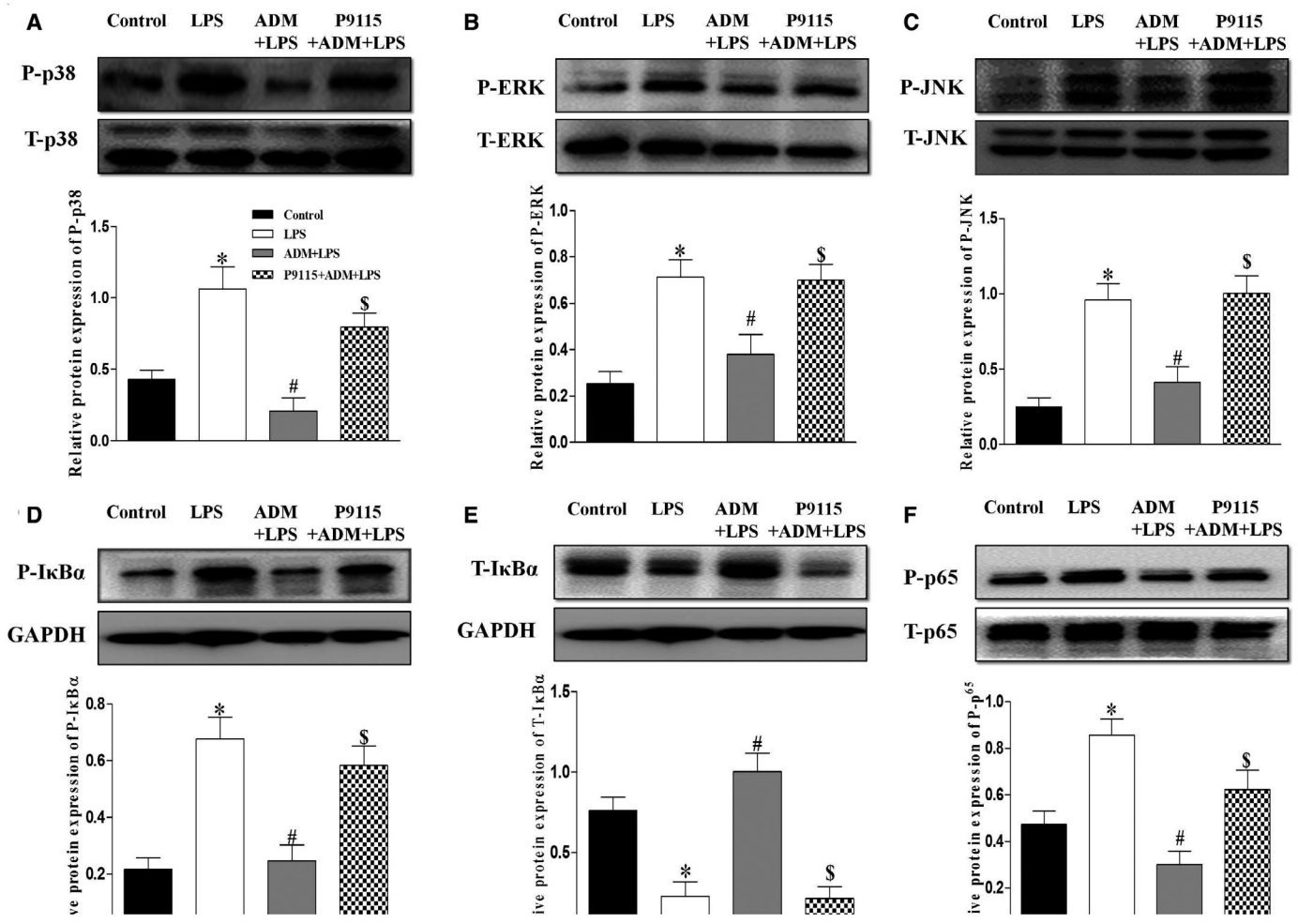

Figure 8 Inhibition of PKA activation with P9115 (10^{-7} M) suppressed effects of ADM on LPS-induced phosphorylation of MAPK (P-p38, P-ERK1/2, and P-JNK) and NF-κB in the differentiated 3T3-L1 adipocytes. Cells were pretreated for 30 minutes with P9115. Following treatment with ADM for 24 hours, each extract was prepared to determine the levels of (**A-C**) phosphorylated MAPK, (**D,E**) IκBα and total IκBα, and (**F**) p65 by the Western blot method. Total MAPK (p38, ERK1/2, and JNK), p65, and GAPDH were used as an internal control. Each value indicates mean ± SEM; $n = 3$ to 5. $^{*}P < 0.05$ vs. control group. $^{\#}P < 0.05$ vs. LPS group. $^{\$}P < 0.05$ vs. ADM + LPS group.

anti-inflammatory, antioxidant, proangiogenic, and vasodilation effects (10,11). Accumulated evidence has demonstrated that ADM has widely anti-inflammatory effects in cells or animal disease models (30-34). Therefore, ADM may have the ability to inhibit inflammation in WAT. In the present study, we indeed found that ADM not only reduced plasma CRP and TNFα levels but also attenuated inflammation by suppressing the protein expression of inflammatory mediators TNFα, IL-1β, COX-2, and iNOS in WAT of obese rats. Moreover, it inhibited protein expression in adipocytes stimulated by LPS. These results suggested that ADM may be a useful agent for prevention and treatment of obesity-related diseases such as inflammation in WAT in the obese state. Although ADM played an anti-inflammatory role in adipose tissue of obese rats in this study, it may inhibit inflammation in other tissues and organs. Therefore, there is still a possibility that the inhibitory effect of ADM on inflammation in adipose tissue of obese rats is attributable to the improvement of inflammation in other organs or tissues because of the manner of ADM administration.

The MAPK family induces the generation of proinflammatory cytokines. NF-κB, a family of transcription factors, regulates the expression of immune-related cytotoxic factors, including iNOS and COX-2, and proinflammatory cytokines, such as TNFα and IL-1β. NF-κB and MAPK are wellrecognized as targets of anti-inflammatory agents (35,36). In this study, the results showed that ADM inhibited the MAPK (p38, ERK1/2, and JNK) and NF-κB activation through suppression of their phosphorylation in differentiated 3T3-L1 adipocytes. Thus, we consider that the anti-inflammatory effect of ADM is associated with the inhibition of MAPK activation and NF-κB translocation to the nucleus. There may be other signaling pathways such as phosphatidylinositol 3-kinase-Akt involving inflammation in this study, so the mechanisms of ADM's action need to be further explored in future studies. Thus, we consider that the anti-inflammatory effect of ADM partially attributes to the suppression of the activation of MAPK and NF-κB through the receptor-PKA pathway.

ADM and its receptors are located in various tissues (10,11). The effects of ADM can be mediated by ADM interaction with the CRLR and mainly associated RAMP2/3, and the downstream signal is transduced mainly by the cAMP-PKA system (20,21,37). Receptor activation has

been shown to mediate the protective effects of ADM (20,21,37). In the present study, the ADM receptor system was expressed in WAT in controls, and the increased expression was also observed in obese rats or in LPS-stimulated adipocytes, suggesting that the upregulation of the ADM receptor system may be protective against inflammation and that ADM may prevent inflammation through upregulation of its functional receptor system. In this study, the anti-inflammatory effect of ADM was effectively blocked by ADM receptor antagonist ADM22-52 pretreatment, indicating that receptor-mediated signaling may respond to ADM. ADM via its receptor activates cAMP production involving cAMP-PKA signaling in its functional mechanism in a variety of cells (38,39), which is also important for ADM to exert its protective roles in some disease states (21,37,39). Indeed, our results showed that LPS-stimulated activation of MAPK and NF-κB and release of inflammatory mediators were markedly attenuated by ADM receptor antagonist or PKA inhibitor pretreatment, which supports the notion that ADM, via the receptor-PKA signaling pathway, inhibits inflammation in adipocytes. In addition to the anti-inflammatory role of ADM in adipose tissue of obese rats, we also found that ADM application reduced the body weight of obese rats. ADM has promotional effects on anorexia (40,41) and it inhibits gastric emptying and histamine and acid secretion from the stomach (42) that may result in the inhibitory effects on food intake. ADM can also promote lipolysis in adipocytes (43,44). Therefore, the decreased body weight of obese rats may be associated with ADM's anorexigenic effects, the inhibition of gastric emptying and acid secretion, or the promotion of lipolysis. All these need to be further explored. Consequentially, ADM could be interesting as a potential target for the treatment of obesity.

Conclusion

ADM alleviated inflammation in WAT of obese rats and in cell culture induced by LPS, and the anti-inflammatory effects of ADM may be partially attributable to the suppression of the activation of MAPK and NF-κB through the receptor-PKA pathway. ADM may play a very important protective role in pathophysiological states of obesity, which results in inflammation in WAT.O

Acknowledgments

We gratefully acknowledge the generous support of the Collaborative Innovation Center for Cardiovascular Disease Translational Medicine. The data sets used and/or analyzed in this study will be made available by the authors on reasonable request.

Funding agencies: This work was supported by the National Natural Science Foundation of China (81970356 and 81470539).

Disclosure: The authors declared no conflict of interest.

Author contributions: Y-BZ designed the study. H-BD, F-ZW, YK, JS, HZ, and QG performed experiments. Z-ZL and PQ performed the data analysis. Y-BZ drafted the manuscript, and G-QZ critically revised the manuscript.

Supporting information: Additional Supporting Information may be found in the online version of this article.

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