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Identification of protective components that prevent the exacerbation of goose fatty liver: Characterization, expression and regulation of adiponectin receptors

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ABSTRACT

Fat accumulation in the liver is a natural process in goose, which prepares goose for long-distance migration. In contrast to mammalian fatty liver that usually progresses into an irreversible status, steatohepatitis, goose fatty liver can return to normal without obvious pathological damage, suggesting a protective system exists in goose liver. This study was to identify the components of this system. We first focused on goose adiponectin receptor 1 and 2 (*Adipor1/2*) as they have ceramidase activity, and can cleave ceramide, a group of proinflammatory signaling lipid species. Quantitative analysis indicated that tumor necrosis factor alpha (*Tnfα*), a key proinflammatory cytokine, was down-regulated in goose fatty liver by overfeeding. This inhibition of *Tnfα* was accompanied with reduced adiponectin and increased *Adipor1/2* in the adipose tissues and in the livers of the overfed geese, respectively. To investigate the regulation of goose *Adipor2* in the context of fatty liver, we treated goose primary hepatocytes with fatty liver associated factors. Data indicated that *Adipor2* was upregulated by glucose and oleate but not palmitate. Its expression was even suppressed by high level of insulin. The regulation of *Adipor1* by these factors was quite similar to that of *Adipor2* except that glucose did not induce *Adipor1*. Together, these findings suggest the upregulation of *Adipor1/2* may, at least partially, contribute to the inhibition of inflammation in goose fatty liver, and the expression of *Adipor1/2* can be regulated by fatty liver-associated factors.

1. Introduction

Goose liver has an excellent capacity to deposit fat. Landes goose, a specialized breed for the production of fatty liver (*foie gras*), can produce fatty liver 8–10 times heavier than normal liver (Hermier et al., 1994). Besides this marvelous tolerance to hepatic steatosis, goose as a descendant of migratory bird can recover from severe hepatic steatosis without any obvious pathological symptoms. In contrast, non-alcoholic fatty liver disease (NAFLD) in mammalian animals including human is usually accompanied with pathological symptoms including inflammation, cirrhosis, and even cancer (Roberts, 2007). Thus, it is believed that goose has a mechanism protecting its liver from harmful effects related to severe hepatic steatosis. This makes goose a unique model animal for uncovering the protective mechanism, which may provide therapeutic approaches to human and other mammalian NAFLD.

Inflammation features in the progression of human and mouse NAFLD from steatosis into steatohepatitis, a key step defining whether pathological damage occurs (Ahmed et al., 2010; Subramanian et al., 2011). Adiponectin is a cytokine exclusively secreted by adipocytes. Adiponectin and its receptors constitute a complex that plays anti-inflammatory, insulin sensitizing, and anti-apoptotic roles in fatty liver (Holland et al., 2011; Xu et al., 2003). However, the level of adiponectin in the blood, the expression of adiponectin in abdominal adipose tissue, and the expression of adiponectin receptors in the liver are all decreased in obese human or mouse with NAFLD (Arita et al., 2012; Hu et al., 1996; Kaser et al., 2005). As goose may have a unique protective system preventing pathological damages associated with severe hepatic steatosis, it is unclear whether inflammation is inhibited in goose fatty liver and whether adiponectin and its receptors contribute to this inhibition.

To determine whether goose fatty liver is accompanied with inflammation, a well-established model of goose fatty liver was employed in this study. Using this model, we examined the expression of tumor necrosis factor-α (*Tnfα*), an inflammation marker gene, in the livers of the overfed *vs.* normally-fed Landes geese by quantitative PCR. In contrast to the induction of *TNFα* in human NALFD (Alaaeddine et al., 2012; Coulon et al., 2012; Lebensztejn et al., 2010; Pagadala et al., 2012), the expression of *Tnfα* was suppressed in goose fatty liver. To identify the protective components that contribute to the suppression of *Tnfα*/inflammation, we determined the expression of adiponectin in abdominal adipose tissue and the expression of adiponectin receptors (Adipor) in

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the liver. However, the expression of adiponectin was not induced, but both *Adipor1/2* were induced in goose fatty liver. To identify the factors that are responsible for the induction, goose primary hepatocytes were treated with several fatty liver-associated factors (*i.e.* high glucose, fatty acids and insulin). Data indicated that high glucose and oleate could contribute to the induction of *Adipor1/2*. Together, these findings suggest *Adipor1/2* may serve as protective components preventing hepatic inflammation in goose fatty liver, which established a foundation for further investigation on the protective role of *Adipor1/2* in goose fatty liver.

2. Material and methods

2.1. Animals

A batch of 10 healthy male Landes geese (63-d-old) grown under natural conditions were provided by Ruinong Farm (Yangzhou, China). The geese were divided into a control group (n = 5) and a treatment/overfeeding group (n = 5). The overfeeding was performed by a skilled worker. First, the mouth of an individual goose was opened, the tube part of a funnel was then put in the mouth, and finally the diet was force-fed by an electronic machine into the body of the goose through the funnel. Two dietary regimes were applied: the geese in overfeeding group were overfed with a carbohydrate diet consisting of boiled maize (maize boiled for 5 min, supplemented with 1% plant oil and 1% salt), and the geese in control group were allowed *ad libitum* feeding of the boiled maize. Before overfeeding, 1-week pre-feeding was conducted in the geese to increase their adaption to overfeeding. In this week, feed intake was gradually increased to 350 g per day. At 70-d-old, the geese were formally overfed from 5/24/2014 to 6/11/2014. The overfeeding protocol was as follows: in the first 5 d the daily feed intake (3 meals a day) reached 500 g, followed by 800 g per day (4 meals a day) for the following week, and 1200 g per day (5 meals a day) for the remaining days till sacrifice. Average body weights of the normally fed and overfed geese were 3.87 kg and 7.57 kg at sacrifice. The geese were kept in cages with free access to water. The routine husbandry management was performed through this study. Tissues including livers and abdominal adipose tissues were collected from the geese at 89 d of age. All tissues were snap-frozen in liquid nitrogen and stored at −70 °C for later use. Animal protocols were approved by the Yangzhou University Animal Ethics Committee.

2.2. Preparation of goose primary hepatocytes

Hepatocytes were isolated from Landes goose embryos at 23 days of incubation. The protocol for hepatocyte isolation is briefly described as follows: place a goose embryo in a sterilized plate, open body cavity, move the liver out, rinse the liver with physiological saline, mince the liver in a sterilized vial, and digest the minced liver with 3–5 volumes of digestive solution containing 0.1% type IV collagenase (Cat. No. LS004186, Worthington, USA) at 37 °C for 40 min (shake the mixture every 10 min to disperse hepatocytes), then add equal volume of complete culture medium that contains high glucose DMEM (Cat. No. C11995500, GIBCO, USA), 100 IU/mL penicillin (Cat. No. P1400, Sigma, USA), 100 kg/mL streptomycin (Cat. No. P1400, Sigma, USA), 2 mM glutamine (Cat. No. ST083, Beyotime, USA), 10% fetal bovine serum (Cat. No. 10099141, Clark, Australia), and 0.02 ml/L EGF (Cat. No. 315-09-100, Peprotech, UK), subsequently, filter the mixture with sterile nylon net (200 meshes) to discard undigested tissues, spin down hepatocytes from the collected liquid at 22.4 ×*g* (r = 80 mm) for 8 min at room temperature, add 3 volumes of red blood cell lysis buffer (Cat. No. R1010, Solarbio, China) to cell pellet, suspend the hepatocytes and incubate on ice for 10 min, followed by centrifuging the mixture at 22.4 ×*g* (r = 80 mm) for 5 min at room temperature, discard the supernatant and rinse the cell pellet with culture medium, repeat this step twice, finally, suspend the cell pellet with complete culture medium, count the number of cells, dilute the cells with culture medium to 1×10^6 cells/mL, plate 1×10^6 cells per well in 12-well dishes, followed by incubation in 5% CO_2 incubator at 38 °C until treatment. The media was renewed at first 6 h of incubation.

2.3. Treatment of cultured cells with glucose, fatty acids and insulin

After 28 h of incubation at 38 °C, old culture medium was removed, and the isolated goose primary hepatocytes were rinsed with phosphate buffered saline (PBS) for later treatment. For glucose treatment, the stock solution of 500 mM glucose (Cat. No. G7021, Sigma, USA) was made by dissolving 0.45 g into 5 mL complete culture medium at room temperature, followed by mixing certain amount of the stock solution with complete culture medium to make a final solution containing 100 mM or 200 mM glucose, which was used to treat the cells for 14 h. The control was the cells treated with complete culture media alone. As hyperlipidemia is often associated with fatty liver disease, saturated fatty acids (*e.g.* palmitic acid) are generally toxic and unsaturated fatty acids (*e.g.* oleic acid) can suppress this toxicity, we performed palmitate treatment with or without supplemental oleate in goose primary hepatocytes. For palmitate treatment, the stock solution of 100 mM potassium palmitate (Cat. No. P5585, Sigma, USA) was made by dissolving 25.6 mg into 1 mL water supplemented with 10 μL dimethyl sulfoxide (DMSO) (Cat. No. O7501, Solarbio, China) at room temperature, then a working solution of 2.5 mL potassium palmitate was made by mixing 1 μL of stock solution with 39 μL complete culture media that contained 2% fatty acid-free bovine serum albumin (BSA) (Cat. No. HWG23861, Beijing HWRK Chemical Co., Ltd.). Using this working solution, a final solution of culture medium containing BSA-conjugated 0.25 mL or 0.50 mL potassium palmitate was made for treating the cells for 14 h. The control was the cells treated with complete culture media containing 2% BSA plus 10 μL/mL DMSO. Similarly, a final solution of culture medium containing BSA-conjugated 0.25 mM or 0.50 mM sodium oleate (Cat. No. O7501, Sigma, USA) was made for oleate assays. For insulin treatment, 1 mM insulin solution as stock solution was made by dissolving 4.9 mg insulin powder (Cat. No. I5500, Sigma, USA) into 865 μL water plus 10 μL 2% hydrogen chloride (HCl). A certain amount of the stock solution was then diluted with water into 10 μM of the working solution, which was in turn used to make a final solution containing 100 nM or 200 nM insulin by mixing 10 μL of working solution with 1 mL of complete culture medium. The control cells were treated with complete culture media containing 11.6 μL/mL of HCl.

2.4. Total RNA isolation and cDNA synthesis

All the primary hepatocytes were rinsed with PBS twice, followed by harvesting the cells at the end of treatment with 1 mL per well. Total RNA from tissues and cultured primary hepatocytes was isolated using TRIzol Reagent (Cat. No. 15596026, Life, USA) according to the manufacturer's instructions. Using total RNA samples, cDNA was synthesized as previously described (Zhang et al., 2013).

2.5. Cloning and sequencing

AdipoR2 cDNA synthesized from total RNA of Landes geese liver was used as template in polymerase chain reaction (PCR) to amplify coding sequence (CDS) of AdipoR2. Forward and reverse primers were designed from the sequence highly conserved between chicken and duck AdipoR2 mRNAs (GenBank accession numbers: NM_001007854.1 and DQ355029.1). The sequences of the primers are as follows: forward primer, 5′-CCA GCA TCT CAT GAA TGA ACT-3′, and reverse primer, 5′-CTG AAG CCT GGT GTT ACT GCA TC-3′. The PCR amplifications were carried out in a final volume of 20 μL. Each reaction contained 1 μL cDNA (200 ng), 2.5 μL 10 × PCR buffer (Mg2 +), 2 μL dNTP mixture (2.5 mM), 1 μL of each primer (10 μM), 0.2 μL Taq (5 U/L), and 12.3 μL ddH_2O. The PCR reagents including 10 × PCR buffer, dNTP mixture