The Acute Phase Protein Orosomucoid Regulates Food Intake and Energy Homeostasis via Leptin Receptor Signaling Pathway

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ABSTRACT

The acute phase protein orosomucoid (ORM) exhibits a variety of activities *in vitro* and *in vivo*, notably modulation of immunity and transportation of drugs. We found in this study that mice lacking ORM1 displayed aberrant energy homeostasis characterized by increased body weight and fat mass. Further investigation found that ORM, predominantly ORM1, is significantly elevated in sera, liver and adipose tissues from the high-fat-diet (HFD) induced obese mice and *db/db* mice that develop obesity spontaneously due to mutation in the leptin receptor (LepR). 

*iv* or *ip* administration of exogenous ORM decreased food intake in C57BL/6, HFD and leptin-deficient ob/ob mice, which was absent in *db/db* mice and was significantly reduced in mice with Arcuate Nucleus (ARC) LepR knockdown, whereas enforced expression of ORM1 in ARC significantly decreased food intake, body weight and serum insulin level. Furthermore, we found that ORM is able to bind directly to LepR and activate the receptor-mediated JAK2-STAT3 signalling in hypothalamus tissue and GT1-7 cells, which was derived from hypothalamic tumor. These data indicated that ORM could function through LepR to regulate food intake and energy homeostasis in response to nutrition status. Modulating the expression of ORM is a novel strategy for the management of obesity and related metabolic disorders.
Obesity is a condition marked by excess accumulation of body fat that results from an imbalance between calorie intake and energy expenditure. Energy homeostasis in the body is maintained by the integrated actions of multiple factors (1,2), including adipose hormones (such as leptin, adiponectin), gastrointestinal hormones (such as insulin, ghrelin, CCK), and nutrient-related signals (such as free fatty acids). In addition to act on peripheral tissues, they can also influence central circuits in the hypothalamus, brain stem, and limbic system to modulate food intake and energy expenditure (1,3). Notably, the adipose tissue produced leptin is a major regulator of fat, and the level of leptin in circulation is proportional to body fat (4) and is a reflection of long-term nutrition status as well as acute energy balance. Furthermore, leptin deficiency or leptin receptor (LepR) mutation led to hyperphagia, obesity and insulin resistance (5), whereas administration of leptin caused weight loss and improved insulin resistance and hyperglycemia in type 2 diabetes mice (6,7). Patients with leptin deficiency or LepR mutation also developed severe obesity (8,9). It is evident that hypothalamic LepR (10,11) is critical for leptin-mediated regulation of energy metabolism, as impairment of LepR signaling in the hypothalamus selectively resulted in hyperphagia and adiposity (1,12,13).

Orosomucoid (ORM), also known as α1-acid glycoprotein (AGP), is one of the acute phase proteins. There are two isoforms of ORM in human (ORM1 and ORM2), one isoform in rat (ORM), and three isoforms in mouse (ORM1, ORM2 and ORM3) (14). These genes have an identical structure with six exons and five introns. Both in humans and mice, constitutive level of ORM1 is much higher (5-fold) than ORM2, and only ORM1 can be induced by acute-phase
stimuli (15). Although it is mainly synthesized by the liver, many extra-hepatic tissues, including adipocytes, heart and brain, are capable of producing ORM under myriad physiological and pathological conditions (16-19). A variety of activities have been attributed to ORM, which include modulating immunity, carrying drugs, maintaining the capillary barrier, and mediating sphingolipid metabolism (14,20-23). It has been reported that the effects of ORM on macrophages, neutrophils, and liver parenchymal cells are mediated by membrane receptor CCR5, Siglect-5, and hemoglobin β-chain respectively (24-26). Interestingly, increase of serum ORM level has been observed in obese humans, mice and Ossabaw pigs (17,27-29). The increased level is correlated with body mass index (BMI), body fat mass, serum leptin, and fasting plasma glucose level in human (27,30). In addition, adipose ORM level is correlated with adiponectin that regulates glucose level and fatty acid breakdown and is regulated by insulin, high glucose, and free fatty acid in differentiated adipocytes (17,27). These results suggested that ORM might participate in the regulation of energy balance.

In this study, we found alterations of energy homeostasis in mice deficient of ORM1, which accounts for the majority of serum ORM as well as most of the changes induced by acute-phase stimuli (15,31). The aberrant energy homeostasis is characterized by significant elevation in body weight and fat mass, increased serum total cholesterol, fatty liver, and insulin and leptin resistance. We also found that ORM derived from adipose and liver tissues is regulated by short- or long-term nutrition signals, and administration of ORM affects feeding behaviour. Furthermore, we demonstrated that ORM binds to LepR and activates the JAK2-STAT3 pathway
in hypothalamus. Thus, ORM could function as an agonist for leptin receptor and is an important regulator in food intake and energy homeostasis.
RESEARCH DESIGN AND METHODS

Reagents

ORM was purchased from Sigma (St. Louis, MO). BSA was obtained from Boguang Biological Technology (Shanghai, China). IgG was from Beyotime Institute of Biotechnology (Shanghai, China). FITC labelled ORM and BSA were made by Youke Biological Technology (Shanghai, China). Antibodies against ORM (rat) and LepR were purchased from Abcam (Cambridge, UK). Antibodies against JAK, p-JAK, STAT3 and p-STAT3 were from Cell Signaling Technology (Danvers, MA). Antibody against ORM (mouse) was obtained from Genway (San Diego, CA). Antibodies against GAPDH, TUBULIN were from Beyotime Institute of Biotechnology (Shanghai, China). Secondary antibodies conjugated with IRDye 800CW were from Rockland Immunochemicals Inc. (Limerick, PA). The LepR siRNA and its control siRNA were from Santa Cruz Biotechnology (Dallas, Texas, USA). Lentivirus carrying full-length ORM1 or LepR-shRNA was constructed by Shanghai GenePharma Co.,Ltd (Shanghai, China). The sequence used for LepR shRNA is 5’-GCTGAAATTGTCTCAGCTAAC-3’. The 60% high fat diet and standard chow were purchased from Slac (Shanghai, China).

Cell Culture and Transfection

Mouse hypothalamic GT1-7 cells were generously provided by Professor Xiao-Ying Li from Shanghai Clinical Center for Endocrine and Metabolic Diseases in Shanghai Jiaotong University School of Medicine (Shanghai, China). C2C12 cells (mouse muscle myoblasts) were obtained
from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). All cells were incubated at 37℃ in a 5% CO2 incubator. For knockdown studies, these cells were transfected with Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

**Animals**

Eight-week-old male db/db and ob/ob mice were purchased from Slac Laboratory Animal (Shanghai, China). Male C57BL/6 mice (18-22g) and Sprague-Dawley rats (180-200g) were purchased from Sino-British SIPPR/BK Laboratory Animals (Shanghai, China). ORM1 knockout mice were generated as reported previously (32), and were backcrossed 10-times with C57BL/6 mice before they were characterized. All animal experiments were undertaken in accordance with the National Institute of Health’s “Guide for the Care and Use of Laboratory Animals”, and with the approval of the Scientific Investigation Board of the Second Military Medical University.

**RNA Quantification**

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) following manufacturer’s instructions. Real-time quantitative RT-PCR analysis was performed using the SYBR RT-PCR kits (Takara, Otsu, Japan). Primer sequences were shown in Supplementary Table 1.
**Blood Parameters**

Serum levels of ORM, leptin and insulin were detected by ELISA kit according to the manufacture’s instruction. Rat ORM ELISA Kit was obtained from Abcam (Cambridge, MA, UK). Leptin ELISA Kit was bought from R&D Systems (Minneapolis, MN, USA). Insulin ELISA Kit was bought from Millipore (Billerica, MA). Total plasma cholesterol and triglyceride were measured by the Clinical Biochemical Lab in Changhai Hospital (Shanghai, China).

**Food Intake**

The effect of ORM on eating behavior was evaluated by the amount of food intake. For fasting-induced food intake, mice were starved overnight (no drinking limited), and the weight of consumed food at 2 h, 8 h and 24 h was recorded 30 min after tail vein injection of vehicle or ORM (100mg/kg). For spontaneous food intake, mice were intraperitoneally injected with vehicle or ORM (50mg/kg/d) for consecutive 4 or 7 days as indicated, and the weight of daily consumed food was recorded.

**Oil Red O Staining**

Frozen 20-µm-thick mouse liver sections were fixed in 4% paraformaldehyde. After removing formalin and washing again with PBS, slides were incubated with Oil Red O working solution (Sigma-Aldrich, MO, USA) at room temperature for 10 min. Then slides were differentiated in 60% ethanol solution and rinsed 3 times with distilled water. Finally, stain in hematoxylin for 30 seconds and wash thoroughly. Photos were taken under the optical microscope (Olympus,
Proximity Ligation Assay (PLA)

GT1-7 cells or C2C12 cells were treated with ORM (10ug/ml) or vehicle for 3 h and fixed with 4% paraformaldehyde. Cells were incubated with primary antibodies against ORM (1:300, rabbit, Genway, San Diego, CA) and Leptin Receptor (1:50, goat, Santa Cruz Biotechnology, Santa Cruz, CA) overnight. PLA was performed using the Duolink in situ PLA kit (Olink Bioscience, Uppsala, Sweden) with PLA PLUS or MINUS probes for rabbit or goat anti-serum. The nuclei of cells were stained using DAPI (Olink Bioscience).

Immunoblotting and Immunoprecipitation

Cells and tissues were lysed with the Cell & Tissue Protein Extraction Reagent (Kangchen, Shanghai, China) supplemented with a protease inhibitor mixture (Kangchen, Shanghai, China). For immunoblotting, cell and tissue lysates (30-50ug protein) in the supernatant were separated by SDS-PAGE and transferred to nitrocellulose membranes. For immunoprecipitation, cell or tissue lysates (100-200ug) were incubated with 2ug of either polyclonal antibody against ORM (Abcam) or a polyclonal LepR antibody (Abcam) coupled to protein G-sepharose (Invitrogen) overnight at 4°C. Immunoprecipitates were washed and separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were then probed for specific proteins.

Immunohistochemistry (IHC)

Immunohistochemistry was carried out according to the standard protocol using the slides from
brain tissues. After overnight incubation at 4°C with the anti-ORM antibody (1:50, rat, MyBioSource) or anti-LepR antibody (1:50, goat, Santa Cruz), the slides were washed and incubated with Cy3-conjugated anti-rat IgG or Alexa Fluor 488-conjugated anti-goat IgG (Jackson ImmunoResearch) for 2 h at room temperature. Following extensive washing, the nuclei were stained with DAPI. Negative controls were run concurrently, except the antibody dilution buffer was used to substitute the primary antibody.

**Flow Cytometry**

The interaction of ORM with the surface of GT1-7 cells was analyzed using flow cytometry. Briefly, cells were blocked with 5% BSA and incubated with FITC-conjugated BSA, FITC-conjugated ORM (10μg/ml), FITC-conjugated ORM in combination with LepR antibody (mouse blocking peptide, 10μg/ml) (Alpha Diagnostic International, San Antonio, TX, USA), or FITC-conjugated ORM in combination with leptin (10μg/ml, Abcam) for 1 hour, and washed with PBS three times, and then analyzed by flow cytometry.

**Stereotaxic Lentivirus Injection**

Male C57BL/6 mice (25-30g) were fixed in ALC-H motorized digital stereotaxic instrument (Shanghai Alcott Biotech Co. Ltd., Shanghai, China). Lentiviruses expressing ORM1, shRNA silencing LepR, or their corresponding controls were injected into ARC (bilaterally, 0.5μl per side; 10^9 transduction units [TU]/μl) by microliter syringes (Hamilton CO., Reno, NV, USA). The coordinates for lateral ventricular injection were as follows: anterior–posterior (from bregma): −2 mm; dorsal–ventral (from skull surface): −6 mm; and medial–lateral: 0.24 mm. All
the mice with ARC injection were examined via immunofluorescence microscopy to verify the correct placement after experiments were completed. Virus in ARC occasionally spread over other nucleus (usually VMN) in the hypothalamus. Data from mice with proven injection in ARC were collected.

**Intraperitoneal Glucose Tolerance Testing (IPGTT)**

IPGTT was performed according to the protocol recommended by Animal Models of Diabetic Complications Consortium (AMDCC). Mice were fasted for six hours by removal to a clean cage without food at the end of their dark (feeding) cycle. After 6h of fasting, the basal blood glucose derived from tail venous blood was detected. Then 2mg/g body weight of glucose was injected intraperitoneally. Blood samples were collected at 15, 30, 60, and 120 min post-injection to measure blood glucose levels using an OneTouch Ultra glucometer (LifeScan Inc., Milpitas, CA, US).

**Molecular Modeling**

The model of the leptin-binding domain of mouse leptin receptor (LepR-LBD) was built by the Homology Modeling protocol in Discovery Studio version 3.5 (Accelrys Inc., San Diego, CA, USA). The X-ray crystal structure of human LepR-LBD (33) was obtained from the Protein Data Bank (34) (PDB, entry code: 3V6O) and used as the template for model building. They share ~75.7% of sequence identity and good superimposition was obtained between the crystal structure and the constructed model (Supplementary Fig. 1A and B). Totally 20 models were generated. The best model verified by MODELLER and Profiles-3D (Supplementary Fig. 1C)
was chosen as the mouse LepR-LBD structure. The X-ray crystal structure of human ORM (i.e. the alpha-1-acid glycoprotein) was taken from PDB (PDB entry code: 3KQ0) (35). Both structures were then prepared with the “Protein Preparation Wizard” workflow in Maestro version 9.3 (Schrödinger L.L.C., New York, NY, USA). Docking of LepR-LBD with ORM was performed using ZDOCK version 2.3 (36). 2000 outputs of the ORM poses were generated for each docking run. All poses were clustered based on an all-against-all root mean squared deviation (RMSD) matrix.

Statistical Analysis

Data are presented as means ± s.e.m. Statistical analyses were performed using GraphPadPrism. Two-tailed Student’s t tests were used to compare two distinct groups. One-way ANOVA followed by Bonferroni’s test was used to compare more than two groups. Interactions were analysed using two-way ANOVA followed by Bonferroni’s test. P<0.05 was considered statistically significant.
RESULTS

ORM1-deficient Mice Displays Alteration of Energy Homeostasis

In previous study, we generated a strain of ORM1-deficient mice to explore the biological function of ORM1 (Supplementary Fig. 2A-D). Interestingly, they had significantly increased serum insulin level at the age of 4 weeks compared to wild type littermates, although these mice had similar serum leptin, total cholesterol (TC), triglyceride (TG), body weight, fat mass and glucose tolerance (Supplementary Fig. 2E-K). When examined at the age of 24 weeks, ORM1-deficient mice showed markedly elevated serum levels of insulin and leptin (Fig. 1A and B), indicatives of insulin and leptin resistance. TC was also increased in these mice, whereas TG levels were not significantly different between wild type and ORM1-deficient mice (Fig. 1C and D). Noteworthily, compared with wild type mice, ORM1-deficient mice had a significant increase in fat mass (1.32±0.20 vs 2.27±0.27, *p<0.05) and a mild increase in body weight (31.3±0.75 vs 34.6±1.05, *p<0.05) (Fig. 1E and F, and Supplementary Fig. 2L-M). While there were no increases in the weights of heart, kidney, pancreas, and spleen (Supplementary Fig. 2L), the weight of liver was markedly raised, accompanied with increased adipose deposition (Fig. 1G). In addition, ORM1-deficient mice showed impaired glucose tolerance (Fig. 1H). Therefore, deficiency of ORM1 in mice impaired energy homeostasis, which prompted us to further investigate the mechanisms and potential physiological and pathological significance of the ORM1 action.
ORM Expression is Elevated in the Obese Mice

To further clarify the relationship between ORM and obesity, we examined ORM expression in high-fat- diet (HFD) induced obese mice and in LepR-defected db/db obese mice. As shown in Fig. 2, these mice had significantly elevated serum levels of leptin and insulin (Fig. 2A-D) as well as increased ORM levels in serum, liver and subcutaneous adipose tissues (Fig. 2E-J). It is worth noting that, the anti-ORM antibody we used for immunoblotting can’t distinguish the three murine ORM isoforms (ORM1 and ORM2 were single polypeptide chains of 207 amino acids each, and ORM3 contains 206 amino acids). Real-time PCR was then utilized to determine the level of each isoform. As shown in Fig. 2K and L, ORM1 is the most abundant isoform that likely responsible for the majority of ORM increase in the liver and subcutaneous adipose tissues (Fig. 2M-P). Interestingly, it was showed that HFD feeding caused significant increases in both ORM1 and 2 mRNA levels in fat tissue without significant changes in hepatic ORM1 and 2 mRNA levels (17). The differences in HFD feeding period (7 days in the report vs 7 weeks in our study) and fat tissue source (epididymal fat tissue in the report vs subcutaneous fat tissue in our study) might be responsible for the inconsistency. Furthermore, we found that ORM was widely expressed in the brain including hypothalamic area as detected by an antibody that recognizes all forms of ORM (Supplementary Fig. 3A). Isoform-specific qPCR revealed that ORM2 is the major isoform (Supplementary Fig. 3B) in hypothalamus, and the expression of ORMs in the hypothalamus tissue did not change in db/db mice or response to HFD (Supplementary Fig. 3C-D).
**ORM Responds to Acute Nutritional Status**

Short-term acute nutritional changes (24 h fasting and 2 h refeeding) are known to affect serum leptin and insulin levels independent of adiposity (37-39), which illustrated how the body maintains energy homeostasis. We utilized this model to explore the potential role of ORM in the process. Similar to leptin and insulin, serum ORM level significantly decreased in fasted animals, and it increased markedly after refeeding in both mice and rats (Fig. 3A-C and Supplementary Fig. 4). The level of ORM was further assessed in tissues. As shown in Fig. 3D, while ORM expression in liver showed similar pattern of change to that of serum, the amount of ORM in subcutaneous adipose tissue and hypothalamus tissue (Fig. 3E and Supplementary Fig. 3E) did not have such pattern. These data support the notion that liver is responsible for producing ORM under acute nutritional alteration condition.

**ORM Decreases Food Intake Dependent of Hypothalamic LepR**

The close association of ORM and energy homeostasis prompted us to ask whether ORM is involved the regulation of feeding behavior. We examined the effect of purified ORM on food intake in C57BL/6 mice. Tail-vein injection of ORM significantly decreased fasting-induced food intake at 2 h, 8 h and 24 h (Fig. 4A). Intraperitoneal injection of ORM for consecutive 4 days also markedly reduced spontaneous daily food consumption (Fig. 4B). Interestingly, these effects is still found in HFD induced obese mice and ob/ob obese mice that are insulin and/or leptin resistant (Fig. 4C-F), whereas they were not present in LepR-defected db/db mice (Fig. 4G)
and H), indicating the involvement of LepR in the process. The arcuate nucleus (ARC) of the hypothalamus plays a pivotal role in the integration of signals regulating appetite. The ARC lies in close proximity to the median eminence that lacks a complete blood brain barrier (40), and thus is uniquely placed to respond to circulating hormonal signals. It has been shown that the ARC is essential for the regulation of energy balance through LepR (1,41). We therefore asked whether blockade of LepR signalling in hypothalamic ARC affects the action of ORM on energy homeostasis. Lentivirus encoding short hairpin RNA (shRNA) targeting LepR (sh-LepR) or control was injected into hypothalamic ARC stereotaxically (Supplementary Fig. 5A-C). Seven days later, as expected, LepR knockdown in ARC resulted in a significant increase in fasting-induced food intake and spontaneous daily food intake (Fig. 4J and K). While ORM administration still reduced fasting-induced food intake significantly at indicated times in mice injected with control lentivirus, its effect was significantly attenuated in mice treated with sh-LepR (Fig. 4I). ORM-induced reduction in spontaneous daily food intake and serum insulin level was also reversed when LepR knockdown in ARC (Fig. 4J and K). Additionally, injection of lentiviral vector LV-ORM1 into ARC to overexpress ORM1 (Supplementary Fig. 5D and E) resulted in a significant decrease in food intake when compared with control mice (Fig. 4L), and this effect lasted up to 6 weeks. Meanwhile, body weight of these mice and their serum insulin level began to decrease two weeks after LV-ORM1 injection (Fig. 4M and N). These data indicated that ORM acts on hypothalamus to regulate food intake in a LepR dependent manner. Despite its effect in reducing food intake, 4-days ORM treatment did not affect body weight, serum insulin, TC and TG levels in HDF and ob/ob mice (Supplementary Figure 6). Since these
two obese animal models have insulin and/or leptin resistance, it is likely that longer time of ORM administration is needed to further significantly change energy hemeostasis.

**ORM Specifically Binds to the LepR in Hypothalamus**

We then examined whether there exists a direct interaction between ORM and LepR. From rat hypothalamus tissue, ORM/LepR complex could be co-immunoprecipitated, especially after ORM tail-vein injection (Fig. 5A). Use was also made of proximity ligation assays (PLA), which showed that the ORM and LepR interact on membrane of GT1-7 cells (Fig. 5B). Furthermore, flow cytometry analysis found that FITC-labelled ORM bound to GT1-7 cell membrane and the binding was completely inhibited by a LepR blocking peptide (Fig. 5C). Taken together, these data demonstrated that ORM interacts directly with LepR. Intriguingly, this binding was not competitively blocked by leptin (Fig. 5D). Structural studies indicated that leptin binds to the leptin-binding domain (LBD) of LepR, likely in the middle of the LBD’s outer side (33). Based on molecular docking, we predicted that the binding site of ORM is in the middle of the LBD’s inner side (Fig. 5E), which may explain the lack of competition between ORM and leptin. Four clusters were further identified as the most possible binding poses out of totally 2000 output from the docking (Supplementary Table 2). Among them, clusters 1 and 7 provided us with the detailed interactions between ORM and LepR-LBD (Supplementary Fig. 7A and B). They will be utilized as basic models for further mutational analysis.

In mice and humans, only the long isoform (LepRb) has an elongated intracellular domain coupled to downstream signalling cascades (42). Although LepRb is primarily expressed in
hypothalamic regions and arcuate nucleus (ARC) (11), it can also be detected in a broad range of
other cell types in line with the pleiotropic effects of leptin in peripheral tissues (43). We further
observed the interaction of ORM and LepR in rat muscle and mouse myoblast C2C12 cells, and
achieved similar results (Supplementary Fig. 7C and D).

**ORM Acts as an Agonist for LepR to Activate Its Downstream JAK2-STAT3 Pathway in
Hypothalamus**

It has been shown that binding of leptin to LepR activates the associated JAK2 tyrosine kinase
that phosphorylates LepR on tyrosine residues (44,45), which in turn leads to the recruitment and
phosphorylation of STAT3. Disruption of the STAT3 binding site on LepR resulted in
hyperphagia and obesity (46). Therefore, activation of STAT3 is a critical event for
LepR-mediated signalling and a commonly utilized marker. As shown in Fig. 6, ORM treatment
induced a significant increase in the phosphorylation of JAK2 and STAT3 in the hypothalamus of
C57BL/6, HFD, and ob/ob mice (Fig. 6A and B), which was largely attenuated in the db/db mice
(Fig. 6C) and in the mice with ARC LepR knockdown (Fig. 6D). *In vitro*, ORM treatment
induced dose- and time-dependent JAK2/STAT3 phosphorylation in GT1-7 cells (Fig. 6E and F),
and the effects were inhibited by LepR knockdown (Fig. 6G and Supplementary Fig. 8A).
Similarly, the dose- and time-dependent increase of JAK2/STAT3 phosphorylation induced by
ORM in myoblast C2C12 cells was largely blocked when LepR was knocked down
(Supplementary Fig. 8B-E).
**DISCUSSION**

A variety of biological activities have been attributed to proteins of ORM family. It has been shown that cell membrane proteins CCR5, Siglect-5, and HBB can bind with ORM and mediate its action *in vitro* (24-26). However, the signal transduction pathways for these interactions and their biological significance *in vivo* have not been further explored. In the present study, we showed that ORM interacts with LepR specifically both in central and peripheral tissues, and activates its downstream JAK-STAT3 signal pathway. We also found that ORM1-deficiency resulted in changes of energy homeostasis, characterized by adiposity, hyperleptinemia, hyperinsulinemia, hypercholesterolemia and impaired glucose tolerance, whereas administration of exogenous ORM, both centrally and peripherally, reduced food intake, body weight and improved insulin resistance in a LepR-dependent manner. These data indicated that ORM could act as an agonist for LepR. Similar to leptin, ORM activates LepR to regulate food intake and energy homeostasis. Interestingly, it also raised the question why ORM binds to multiple cell membrane receptors. As suggested by the finding that the interaction with CCR5 was partially due to its glycosylation (24), it is likely that the heavy glycosylation of ORM might also contribute to its binding with LepR and other receptors.

The ‘negative-feedback’ model of energy homeostasis has been well established. Circulating signals inform the brain of changes in body fat mass. In response to this input, the brain mounts adaptive adjustments of energy balance to stabilize fat storage. Leptin and insulin are two well-known negative-feedback signals that circulate at levels proportional to body fat content
and can act on the brain to promote weight loss (1). Our data showed that ORM is regulated by nutritional conditions and activates the LepR pathway in the hypothalamus to affect food intake, indicating that it could be another negative-feedback signal and plays an important role in energy homeostasis (Fig. 7). Due to the existence of hypothalamic ORM, it raised the question whether central ORM is also important in the regulation of metabolism. We found ORM2, not ORM1, is the major isoform of ORM in hypothalamus, and there was no response of central ORM to short- or long-term nutritional signals, indicating that it may not be responsible for ORM-induced change of energy homeostasis under these conditions.

In the present studies, we found, in addition to the fat tissue, liver is also the main organ sensing long-term nutritional state, such as high-fat-diet and obese, to produce ORM. ORM is a dual signal both from adipose and gastrointestinal tract. Interestingly, it is the liver, not the fat tissue, that sensed short-term nutritional signal, with ORM decreased after the fasting and increased after refeeding. The ORM derived from gastrointestinal tract is more important for the regulation of food intake in response to acute nutritional signals. This may be related to the specific regulation of liver in protein production. The liver can respond to various stresses and induce the quick production of acute phase proteins, while ORM is one of acute phase proteins mainly produced by the liver (47). In addition, liver FXR (hepatic nuclear bile acid receptor farnesoid X receptor) may be involved in the quick regulation of ORM in response to acute nutritional signal. FXR regulates bile acid homeostasis, while bile acid secretion is affected by immediate food intake (48). ORM is reported to be a direct target gene of hepatic FXR, and hepatic FXR deletion
in mice affects the expression of ORM (48). Fasting and refeeding may promptly affect bile acid secretion, which therefore decrease or increase ORM expression in liver via FXR. Interestingly, vertical sleeve gastrectomy (VSG) is at present the most effective therapy for the treatment of obesity, while the mechanism remains largely unclear. Recently, bile acids and FXR signalling is reported to be an important molecular target for the effects of VSG. In the absence of FXR, the ability of VSG to reduce body weight and improve glucose tolerance is substantially reduced (49). Whether ORM, the downstream target of FXR, is involved in the role of VSG is worthy of being explored.

It should be noted that, although ORM-induced phosphorylation of STAT3 was largely impaired when LepR deficient or knockdown, there still exists the activation of STAT3 pathway, suggesting other unknown receptor involved. Evidences showed that ORM might bind to the macrophages via CCR5 (24), which is also expressed in the hypothalamus and skeletal muscle. As a member of cytokine receptor, CCR5 activation also results in STAT3 phosphorylation in T cells (50). In addition, CCR5 deficiency suppressed lung tumor development through the inhibition of nuclear factor-kappaB/STAT3 pathways (51). Therefore, the remained phosphorylation of STAT3 triggered by ORM might be the results of CCR5 signalling. Interestingly, while we found the effect of ORM on decreases serum insulin levels (most likely the result of reducing food intake and weight loss) is LepR-dependent, Lee et al reported that ORM improved glucose tolerance (OGTT test) and increased the sensitivity to insulin in decreasing blood sugar (ITT test) in the db/db mice. These results indicate that ORM affects insulin action at
least at two levels, controlling the food intake and body weight with subsequent improvement of insulin resistance via central LepR pathway, and increasing peripheral insulin sensitivity in a LepR-independent pathway. Additional receptors such as CCR5 may also be involved in this process, since it was reported that CCR5 activation enhanced glucose uptake in activated T cells, and our previous studies found that ORM increased muscle glycogen content via the CCR5 pathway (32,52). One of our on-going efforts is analyzing the ORM1 and LepR interaction through site-directed mutagenesis.

ORM also showed a variety of functions in peripheral tissues. It has been reported that ORM1 increases glucose uptake activity in 3T3-L1 adipocytes, and relieves hyperglycemia-induced insulin resistance as well as TNFα-mediated lipolysis in adipocytes (17). Accordingly, ORM improved glucose and insulin tolerance in obese and diabetic db/db mice, and could protect adipose tissue from excessive inflammation and subsequent metabolic dysfunction (17). We further found ORM also interacted with LepR in peripheral skeletal muscle, and activated the JAK2-STAT3 signalling. Skeletal muscle, together with liver and adipose tissue, are the tissues with great metabolic activity, which may constitute important targets for ORM in peripheral tissue and may be involved in the regulation of insulin sensitivity as well as glucose and lipid metabolism. The function of ORM in peripheral tissues needs further investigation. Additionally, it has been shown that the level of ORM2 is increased under certain stress condition (19). Therefore, the function of central ORM and other isoforms of ORM, which are not found affected by nutritional signals, remains to be further investigated.
In summary, we found that the expression ORM, one of the acute phase proteins, is regulated by long- or short-term nutritional signals, and ORM affects food intake and energy homeostasis through activating the LepR pathway in the hypothalamus. Thus, it is a negative-feedback molecule in energy homeostasis and a novel target for the management of obesity and related metabolic disorders.
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Duality of Interest. No potential conflicts of interest relevant to this article were reported.
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Figure legends

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**Figure 6**—ORM acts as an agonist for LepR to activate its downstream JAK2-STAT3 pathway in hypothalamus. (A-C) Representative western blot of p-STAT3/STAT3, p-JAK2/JAK2 in the hypothalamus of C57BL/6, HFD, ob/ob or db/db mice treated as described in Figure 4 B, D, F and H (n=6 per group). (D) Representative western blot of p-STAT3/STAT3, p-JAK2/JAK2 in the hypothalamus of mice treated as described in Fig. 4J (n=6 per group). (E-G) Representative western blot of p-STAT3/STAT3, p-JAK2/JAK2 in mouse GT1-7 hypothalamic cell line treated with the indicated doses of ORM for 3 h (E, n=3), 10 µg/ml ORM for the time indicated (F, n=3), or 10 µg/ml ORM or vehicle for 3 h in the presence or absence of LepR RNAi (G, n=3). Western blots are representative of three independent experiments. Quantification data were shown as a
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180x249mm (300 x 300 DPI)
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Online Supplementary Materials

The Acute Phase Protein Orosomucoid Regulates Food Intake and Energy Homeostasis via Leptin Receptor Signaling Pathway

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Dingfeng Su,¹ & Xia Liu¹

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Shanghai 200433, China

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³Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China
**Supplementary Table 1.** Primer sequences utilized for RNA quantification

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### Supplementary Table 2. Docking scores of four major clusters of ORM conformations on LepR-LBD.

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*a The ID of the cluster that the pose is assigned to.

*b The number of poses in Top 100 poses.

*c The size of the cluster that the docked protein pose belongs to.

Totally 2000 output poses were clustered into 100 groups based on RMSD values and each pose was assessed by ZDock Score and ZRank Score. ZDock Score is the shape complementarity score and ZRank Score is the energy of the docked poses calculated by the ZRANK rescoring method. On the basis of the analysis on the Top 100 poses, four clusters were identified as the possible binding poses of ORM on LepR-LBD. As shown in Supplementary Table 2, Cluster 1 has the largest number of poses, and both ZDock Score and ZRank Score of Cluster 1 are relatively higher than others. In addition, because Cluster 7 has the highest ZDock and ZRank Scores in all 2000 poses, the poses in this cluster may be considered as the active pose of ORM.
Supplementary Figure 1. Molecular Modeling. (A) The sequence alignment of mouse and human LepR-LBD. They share ~75.7% of sequence identity (B) Good superimposition of the crystal structure of human LepR-LBD (cyan) (PDB entry code: 3V6O, chain B) with mouse LepR-LBD model (magenta) built by homology modeling. (C) Ramachandran Plot of mouse LepR-LBD model (Red words represent Trp556 and Val586 in generally allowed region).
**Supplementary Figure 2.** Identification and characteristic of ORM1 knockout mice. (A) Genotyping of ORM1 knockout mice. ORM+/+:526bp (P1/P3); ORM-/- 598bp (P2/P3). P1: 5’- actgtccctetatgGTAAGCACC - 3’, P2: 5’- CTGAGCCCAGAAAGCGAAGGA -3’, P3: 5’-CACCCTGGCACCGGATATTCC -3’. (B-D) q-PCR detection of ORM1, ORM2 or ORM3 mRNAs in liver (B), SAT (C) or muscle (D) derived from 4-week-old male ORM+/+ or ORM-/- mice (n=6 per group). The mRNA level of ORM1 in ORM+/+ mice was calculated as 100%. (E-K) Serum level of (E) insulin, (F) leptin, (G) total cholesterol (TC), (H) triglyceride (TG), (I) body weight, (J) organ weight and fat mass, and (K) blood glucose level after IPGTT in 4-week-old male ORM1 +/+ (n=12) or ORM1 +/- mice (n=7). (L) Organ weight and fat mass in 24-week-old male ORM1+/+ (n=5) or ORM -/-mice (n=11). Inguinal fat: ing; Perirenal fat: peri; Mesenteric fat: mes; Epididymal fat: epi. (M) Body weight distribution in 24-week-old male ORM1+/+ (n=13) or ORM -/-mice (n=20). Data are presented as means ± s.e.m. *p<0.05, **p<0.01 by Student’s t test.
Supplementary Figure 3. ORM distribution and response to nutritional signals in the hypothalamus. (A) Representative immunofluorescence image of ORM in mouse brain tissues (n=3). Bar=50µM. (B) q-PCR detection of relative ORM1, ORM2 or ORM3 mRNAs in hypothalamus derived from C57BL/6 mice (n=6). The mRNA level of ORM1 was calculated as 100%. (C-D) Representative of western blot of ORM in hypothalamus from HFD (C, n=12-13 per group) or db/db mice (D, n=8 per group). (E) Representative of western blot of ORM in hypothalamus derived from C57BL/6 mice after fasting (starving) for 24 hours and refeeding for 2 hours (n=6 per group). Data are presented as means ± s.e.m. *p<0.05, **p<0.01 by one-way ANOVA with Bonferroni’s test.
Supplementary Figure 4. ORM responds to acute nutritional status in rats. (A-C) Serum leptin (A), insulin (B) and ORM (C) level in SD rats after fasting for 36 hours and refeeding for 6 hours (n=6 per group). Data are presented as means ± s.e.m. *p<0.05, **p<0.01 by one-way ANOVA with Bonferroni’s test.
Supplementary Figure 5. Verification of the effect of LepR interference or ORM1 overexpression in ARC. (A) C57BL/6 mice were stereotaxic injected with lentivirus into arcuate nucleus (ARC). One week later, C57BL/6 mice were sacrificed. Representative image of infected neurons with green fluorescent protein (GFP) in ARC was shown. Bar=50µM. (B-C) ARC was injected with lentivirus carrying LepR shRNA (sh-LepR) or matched control (sh-control) in C57BL/6 mice. One week later, the expression of LepR in ARC was assessed by q-PCR (B) and immunofluorescence (C). Bar=20µM. n=6 per group. (D-E) ARC was injected with lentivirus carrying ORM1 (LV-ORM1) or matched control (LV-GFP) in C57BL/6 mice. One week later, the expression of ORM1 in ARC was assessed by q-PCR (D) and immunofluorescence (E). Bar=20µM. n=6 per group. Negative controls were run concurrently, except the antibody dilution buffer was used to substitute the primary antibody. Data are presented as means ± s.e.m. **p<0.01 by Student’s t test.
Supplementary Figure 6. The effect of ORM on the energy homeostasis in HFD and ob/ob mice. (A-D) HFD mice were treated as described in Figure 4D, (A) serum insulin, (B) TC, (C) TG and (D) body weight were detected. n=12 per group. (E-H) Ob/ob mice were treated as described in Figure 4F, (E) serum insulin, (F) TC, (G) TG and (H) body weight were detected. n=8 per group.
Supplementary Figure 7. ORM binds to the LepR. (A and B) Two most possible interaction models of ORM with LepR-LBD, and key residues to form interactions between them (ORM: purple and light blue; LepR-LBD: green). Top 1 pose in Cluster 1 (A); Top 1 pose in Cluster 7 (B). (C) Representative co-immunoprecipitation assay of the interaction of ORM and LepR in the muscle lysates of rats (n=3). IP: immunoprecipitation; IB: immunoblotting. (D) Representative immunofluorescence images of mouse muscle cell line C2C12 treated with vehicle or 10µg/ml of ORM for 3 h (n=3). Each red spot represents an ORM-LepR interaction. Nuclei were stained with DAPI. Bar=20µM.
**Supplementary Figure 8.** ORM activates JAK2-STAT3 signaling in the mouse C2C12 muscle cell line via LepR. (A) GT1-7 cells were transfected with control siRNA or LepR siRNA for 48h. The expression of LepR was assessed by q-PCR (left, n=3 per group) and immunoblotting (right, n=3 per group). (B) C2C12 cells were transfected with control siRNA or LepR siRNA for 48h. The expression of LepR was assessed by q-PCR (left, n=3 per group) and immunoblotting (right, n=3 per group). For A and B, data are presented as means ± s.e.m. **p<0.01 by Student’s t test. (C-E) Representative western blot of p-STAT3/STAT3, p-JAK2/JAK2 in mouse C2C12 muscle cell line treated with the indicated doses of ORM for 3 h (C), 10 µg/ml ORM for the time indicated (D), or 10 µg/ml ORM or vehicle for 3 h in the presence or absence of LepR RNAi (E). For C-E, western blots are representative of three independent experiments. Quantification data were shown as a ratio of phosphorylated protein vs total protein, and the ratio of vehicle treatment in each group was regarded as 100%. Data are presented as means ± s.e.m. For C and D, *p<0.05, **p<0.01 vs. control by one-way ANOVA with Bonferroni’s test. For E, n.s., not significant, **p < 0.01 by two-way ANOVA with Bonferroni’s test. Interaction for pSTAT3/STAT3, F=398, ##P< 0.01; Interaction for pJAK2/JAK2, F=128.8, ##P< 0.01.
Supplementary Figure 1

A

B

C

99x76mm (300 x 300 DPI)
Supplementary Figure 2

A

B

C

D

E

F

G

H

I

J

K

L

M

189x225mm (300 x 300 DPI)
Supplementary Figure 3

A

negative control  cy3  DAPI  Merge

anti-ORM

B

Relative mRNA level

ORM1  ORM2  ORM3

hypothalamus

C

hypothalamus

ORM  ORM  ORM

GAPDH  GAPDH  GAPDH

Con  HFD  C57BL/6  db/db  con  fasting  refeeding

80x45mm (300 x 300 DPI)
Supplementary Figure 4

A  B  C

Supplementary Figure 4

A  B  C

49x22mm (300 x 300 DPI)
Supplementary Figure 6

A  

HFD  

B  

HFD  

C  

HFD  

D  

HFD

0  0.5  1  1.5  2  2.5  3  3.5  4

body weight (g)

E  

serum insulin (ng/ml)  

F  

serum insulin (ng/ml)  

G  

serum insulin (ng/ml)  

H  

ob/ob  

body weight (g)

day 0 1 2 3 4

90x54mm (300 x 300 DPI)
Supplementary Figure 7

A

B

C

D

C2C12 cells

PLA

DAPI

Merged

IP: muscle

IgG ORM IgG LepR

IB ORM LepR

Negative control

Basal state

ORM treatment

175x251mm (300 x 300 DPI)
Supplementary Figure 8

A) GT1-7 cells

B) C2C12 cells

C) C2C12 cells

D) C2C12 cells

E) C2C12 cells

170x199mm (300 x 300 DPI)