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Major Urinary Protein-1 Increases Energy Expenditure and Improves Glucose Intolerance through Enhancing Mitochondrial Function in Skeletal Muscle of Diabetic Mice*[S]

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Xiaoyan Hui[‡], Weidong Zhu[§¶], Yu Wang[¶‖], Karen S. L. Lam[§¶], Jialiang Zhang[§¶], Donghai Wu[**], Edward W. Kraegen[‡‡], Yixue Li[‡], and Aimin Xu[§¶‖1]

From the [‡]Key Laboratory of Systems Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Graduate School of the Chinese Academy of Sciences, Shanghai 200031, China, [§]Department of Medicine, [¶]Research Center of Heart, Brain, Hormone, and Healthy Aging, and [‖]Department of Pharmacology and Pharmacy, The University of Hong Kong, Hong Kong, China, [**]Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510663, China, and [‡‡]Garvan Institute of Medical Research and School of Medical Sciences, University of New South Wales, Sydney, New South Wales 2010, Australia

Major urinary protein-1 (MUP-1) is a low molecular weight secreted protein produced predominantly from the liver. Structurally it belongs to the lipocalin family, which carries small hydrophobic ligands such as pheromones. However, the physiological functions of MUP-1 remain poorly understood. Here we provide evidence demonstrating that MUP-1 is an important player in regulating energy expenditure and metabolism in mice. Both microarray and real-time PCR analysis demonstrated that the MUP-1 mRNA abundance in the liver of *db*/*db* obese mice was reduced by ~30-fold compared with their lean littermates, whereas this change was partially reversed by treatment with the insulin-sensitizing drug rosiglitazone. In both dietary and genetic obese mice, the circulating concentrations of MUP-1 were markedly decreased compared with the lean controls. Chronic elevation of circulating MUP-1 in *db*/*db* mice, using an osmotic pump-based protein delivery system, increased energy expenditure and locomotor activity, raised core body temperature, and decreased glucose intolerance as well as insulin resistance. At the molecular level, MUP-1-mediated improvement in metabolic profiles was accompanied by increased expression of genes involved in mitochondrial biogenesis, elevated mitochondrial oxidative capacity, decreased triglyceride accumulation, and enhanced insulin-evoked Akt signaling in skeletal muscle but not in liver. Altogether, these findings raise the possibility that MUP-1 deficiency might contribute to the metabolic dysregulation in obese/diabetic mice, and suggest that the beneficial metabolic effects of MUP-1 are attributed in part to its ability in increasing mitochondrial function in skeletal muscle.

The liver is the primary organ for carbohydrate and lipid metabolism, including gluconeogenesis, glycogenesis, cholesterol biosynthesis, and lipogenesis (1, 2). These metabolic events in the liver are tightly controlled by several pancreatic hormones including insulin and glucagon. In addition, the liver itself is one of the largest endocrine organs in the body, secreting numerous humoral factors involved in the regulation of systemic glucose and lipid homeostasis. The importance of the liver-derived humoral factors in maintaining glucose metabolism is highlighted by the observation that glucose uptake by skeletal muscle is severely impaired by surgical or pharmacological blockade of hepatic parasympathetic nerves (3). In the past several years, a number of liver-derived humoral metabolic factors, including bone morphogenetic protein-9 (BMP-9) (4), fibroblast growth factor 21 (FGF21) (5–7), retinol-binding protein 4 (RBP4) (8, 9), adropin (10), and angiopoietin-like proteins (Angptl) 3, 4, and 6 (11–13), have been identified, and their roles in glucose and lipid metabolism have been characterized in great detail. Noticeably, BMP-9, FGF21, and Angptl6 exhibit potent insulin-sensitizing and glucose-lowering effects in animal models, and they have been proposed as potential candidates for the treatment of insulin resistance and type II diabetes (4, 6, 7, 13).

To search for novel liver-derived secretory factors involved in the regulation of glucose homeostasis, we used microarray analysis as a global screening for systematic identification of genes differentially expressed in the liver of C57BLKS *db*/*db* mice (a genetically inherited diabetic mouse model that is characterized by severe insulin resistance and hyperglycemia) and their lean littermates. We found that the mRNA level of mouse major urinary protein-1 (MUP-1)[2] was markedly down-regulated in *db*/*db* mice, and the change was largely normalized upon treatment with the PPARγ agonist rosiglitazone. MUP-1 is a small molecular weight secreted protein abundantly expressed in the liver (14). Its expression in the liver is enhanced by administration of the hepatotoxic agent dimethylnitrosamine (15) but is reduced by interleukin 6-induced acute phase

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 and Tables 1 and 2.

[1] To whom correspondence should be addressed: Dept. of Medicine, The University of Hong Kong, L8-40, New Laboratory Block, 21 Sassoon Rd., Hong Kong, China. Tel.: 852-28199754; Fax: 852-28162095; E-mail: amxu@hkucc.hku.hk.

[2] The abbreviations used are: MUP, major urinary protein; PPARγ, peroxisome proliferator-activated receptor γ; PGC1α, PPARγ coactivator 1α; GSK3β, glycogen synthase kinase 3β; ELISA, enzyme-linked immunosorbent assay.

response in mice (16). Like other members of the MUP family, MUP-1 has been proposed to act as a pheromone-binding protein in urine (17), thereby accelerating puberty and promoting aggressive behavior in male mice. However, the precise functions of MUPs have yet to be determined.

MUP-1 belongs to the lipocalin superfamily, the members of which share a common tertiary structure with a cup-shaped hydrophobic ligand binding pocket surrounded by an eight-stranded β-barrel (18, 19). This structure confers upon lipocalins the ability to bind and transport a wide variety of small lipophilic substances, including fatty acids, cholesterols, prostaglandins, and pheromones. Noticeably, several members of the lipocalin family, including RBP4, lipocalin-2, and adipocyte fatty acid-binding protein (A-FABP), have recently been shown to be important mediators of obesity-related insulin resistance and glucose intolerance (8, 20–22). Unlike MUP-1, the expression of RBP4, lipocalin-2, and A-FABP are elevated in obesity and diabetes (9, 20, 23).

In this study, we investigated the metabolic role of MUP-1 in mice. Our results demonstrated that MUP-1 was abundantly present in the circulation. In genetic and dietary obese mouse models, the serum and urine concentrations of MUP-1 were remarkably decreased. Replenishment of recombinant MUP-1 led to improved glucose tolerance and insulin sensitivity, as well as increased energy expenditure and locomotor activity in *db*/*db* diabetic mice. Our data suggest that MUP-1 not only serves as a circulating biomarker, negatively correlated with obesity-related metabolic disorders, but also plays an active role in regulating energy homeostasis and insulin sensitivity in mice.

EXPERIMENTAL PROCEDURES

Animal Studies—C57BL/6J mice and C57BKS *db*/*db* diabetic mice were housed in a room under controlled temperature (23 ± 1 °C), with free access to water and standard mouse chow (LabDiet 5053, LabDiet, Purina Mills, Richmond, IN). To establish a dietary obese mouse model, 4-week-old male C57BL/6J mice were fed a 19.33 kJ/g high fat diet (D12451, Research Diet, New Brunswick, NJ) containing 45 Kcal % fat, 20 Kcal % protein, and 35 Kcal % carbohydrate for 10 weeks as described previously (24). For the intraperitoneal glucose tolerance test, mice that were fasted overnight (16 h) were given a glucose load by intraperitoneal injection (1 g of glucose/kg of body weight). For the insulin tolerance test, mice were starved for 6 h and then injected intraperitoneally with insulin (0.5 unit/kg of body weight). For the pyruvate tolerance test, mice were starved for 16 h and were then injected intraperitoneally with pyruvate (2 g/kg) dissolved in saline. Blood glucose levels were determined in tail blood every 30 min for 2 h using the Ascensia Elite XL blood glucose meter (Bayer HealthCare).

To evaluate the metabolic effects of MUP-1, the recombinant MUP-1 protein was delivered into the mice using Alzet™ osmotic pumps (DURECT Corp., Cupertino, CA) as described previously (25). All experiments were conducted under our institutional guidelines for the humane treatment of laboratory animals.

Indirect Calorimetry—Mice were housed individually in metabolic chambers maintained at 22 °C on a 12-h light/12-h dark cycle with lights on at 7 a.m. Metabolic measurements (food intake, oxygen consumption, respiratory exchange rate, and core body temperature) were recorded continuously using a comprehensive laboratory animal monitoring system (CLAMS, Columbus Instruments). Spontaneous locomotor activity was monitored at the same time as the indirect calorimetry, continuously over the measurement period by infrared beam sensors, separated by 0.54 cm from each other.

Expression and Purification of Recombinant MUP-1 in Escherichia coli—cDNA from the male mouse liver was used as the template for cloning the gene encoding full-length MUP-1, which does not include the secretory signal peptide (amino acids 1–18). The primer sequences used for cloning were 5′-CCGCTGGATCCGAAGAAGC TAGTTCTACGG-3′ (forward) and 5′-ACTGTC TCGAGTCATTCTCGGGCCTG-GAGG-3′ (reverse), respectively. After digestion with the restriction enzymes BamHI and XhoI, the DNA fragment was purified and subcloned into pPROEX-Htb bacterial expression vector (Invitrogen). The gene fragment was inserted in-frame to an N-terminal His_6 tag and was verified by DNA sequencing.

The construct was transformed into *E. coli* BL21(DE3)-codon plus RILP-competent cells for expression of recombinant MUP-1, which was purified by using an Ni^{2+}-nitrilotriacetic acid column as described previously (22). Endotoxin was removed using a Detoxi-Gel™ endotoxin removal kit (Pierce) according to the manufacturer's instructions.

Production of Polyclonal Antibody against Mouse MUP-1 in Rabbits—The anti-mouse MUP-1 polyclonal antibody was generated by immunization of New Zealand female rabbits using recombinant MUP-1 as described (26). Anti-mouse MUP-1 IgG was purified from the serum of immunized rabbits with protein G beads followed by affinity chromatography with recombinant mouse MUP-1 as the ligand.

Measurement of Serum and Urinary MUP-1 by In-house ELISA—Affinity-purified anti-MUP-1 IgG was biotinylated with a sulfo-NHS-biotin kit (Pierce), and the biotinylated IgG was used as the detection antibody. Unlabeled anti-MUP-1 IgG was used as the capture antibody for coating a 96-well microtiter plate overnight at 4 °C at a concentration of 1 μg/ml. Mouse serum samples were diluted in a buffer containing 10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, 1% bovine serum albumin, pH 7.4. Then 100 μl of the diluted samples or recombinant MUP-1 standards were applied to each well, and the mixture was incubated at room temperature for 1 h. The plates were washed three times with a wash buffer (10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, 0.1% v/v Triton X-100, pH 7.4) and then incubated with 100 μl of the detection antibody (0.15 μg/ml) for 1 h at room temperature. After washing three times, the wells were incubated with streptavidin-conjugated horseradish peroxidase for 20 min and subsequently reacted with tetramethylbenzidine reagent for 15 min. 50 μl of 0.5 M H_2SO_4 was added to each well to stop the reaction, and the absorbance was measured at 450 nm.

Enzyme Activity Assays—Frozen adipose tissue, muscle, or liver samples were homogenized in 50 mM Tris-HCl, 1 mM EDTA, and 0.1% Triton X-100, pH 7.2. Citrate synthase activity was measured in a reaction mixture containing 100 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 1 mM EDTA, 0.2 mM dithio-bis(2-nitrobenzoic acid), 0.3 mM acetyl CoA, and 0.5 mM oxaloace-

tate. The rate of change in absorbance was monitored at 412 nm. The activity of cytochrome *c* oxidase was analyzed by measuring the oxidation of reduced cytochrome *c* with a commercially available cytochrome *c* oxidase assay kit (Sigma) as described (27).

Extraction of Total RNA and Quantitative Real-time PCR—Total RNA was isolated from various tissues of mice using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed with an ImProm-II™ reverse transcription kit (Promega, Madison, WI). Quantitative real-time PCR was performed using GreenER™ qPCR Supermix with specific primers (Invitrogen) or TaqMan Master Mix with Assay-On-Demand™ primers and probes (Applied Biosystems, Foster City, CA). The reactions were carried out on a 7000 Sequence Detection System (Applied Biosystems). The mitochondrial DNA (mtDNA) copy number was quantified by comparing the ratio of cytochrome *b* (mitochondrial marker) to the genomic β-actin DNA quantity as described (27). The sequences of the primers used for quantification of each gene and mtDNA are listed in supplemental Table 1.

Western Blotting—Mice serum or recombinant MUP-1 was resolved by15% SDS-PAGE followed by electrophoretic transfer to a polyvinylidene difluoride membrane (GE Healthcare). The membrane was blocked with 10% (w/v) milk and probed with a primary antibody against mouse MUP-1 at 4 °C overnight. After incubation with a horseradish peroxidase-conjugated rabbit anti-sheep IgG antibody, the proteins were visualized with enhanced chemiluminescence reagents (GE Healthcare).

Statistics—All of the data obtained in this study are represented as means ± S.D. The statistical calculations were performed with the Statistical Package for the Social Sciences, version 11.5, software package (SPSS, Inc., Chicago, IL). Differences between groups were determined by an unpaired two-tailed Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

MUP-1 Expression in the Liver Is Markedly Decreased in db/db Diabetic/Obese Mice and Is Up-regulated after Treatment with Rosiglitazone or Refeeding—To identify novel genes differentially expressed under diabetic/obese conditions, we used microarray analysis for global comparison of expression profiles in the liver tissue between lean mice and C57BLKS *db*/*db* mice (a genetically inherited obese mouse model with frank diabetes). Our results showed that the expression of a gene encoding MUP-1, a secreted protein comprising of 180 amino acid residues (GenBank™ accession number NM_0311881), was markedly decreased by ~30-fold in the liver tissue of *db*/*db* obese/diabetic mice compared with their lean littermates, whereas treatment of *db*/*db* diabetic mice with the PPARγ agonist rosiglitazone partially reversed this change. We further confirmed this microarray data by using real-time PCR analysis (Fig. 1*A*).

Tissue distribution analysis showed that MUP-1 was synthesized predominantly in the liver (supplemental Table 2). A much lower level of MUP-1 mRNA expression was also detected in heart, epididymal fat, and brown adipose tissue but not in several other tissues examined (skeletal muscle, kidney, brain, and spleen). The decreased MUP-1 mRNA expression in *db*/*db* mice was observed mainly in liver tissue. Furthermore, the mRNA expression of MUP-1 was altered upon changes in nutritional status. After overnight fasting, the mRNA level of MUP-1 was decreased by 50%, whereas its expression was markedly up-regulated by 1 h after refeeding (Fig. 1*B*).

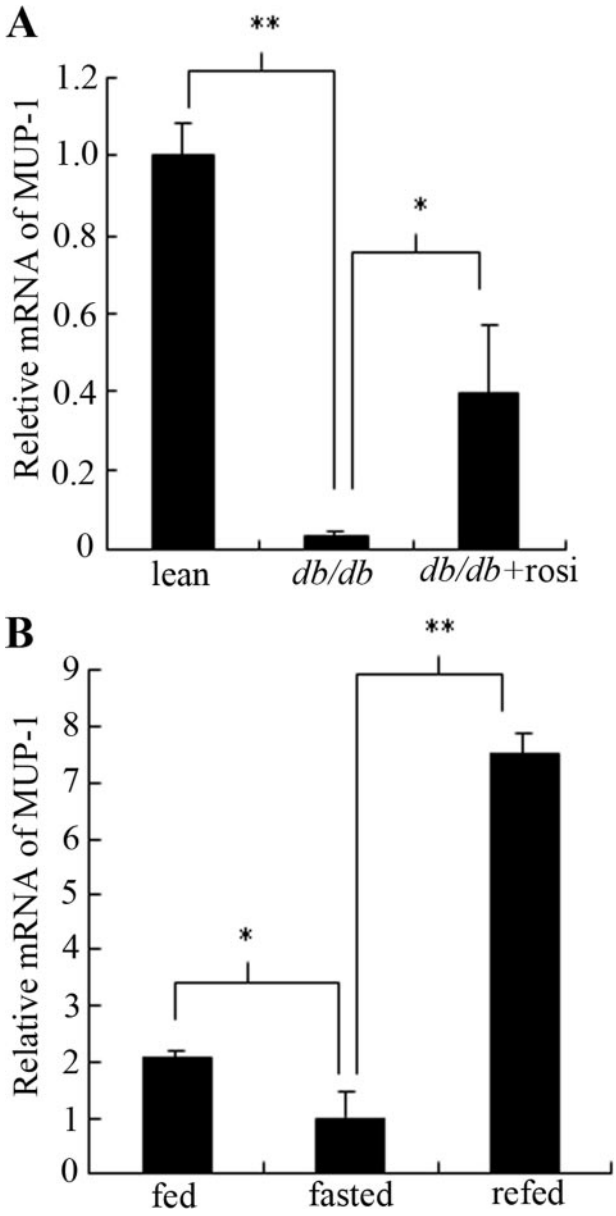

FIGURE 1. **The mRNA of MUP-1 in the liver is altered in obese/diabetic condition and upon nutritional changes.** *A*, the abundance of MUP-1 mRNA was markedly down-regulated in the livers of *db*/*db* mice and up-regulated upon rosiglitazone treatment. 10-week-old male *db*/*db* mice were treated with saline as vehicle control or rosiglitazone (*rosi*) by oral gavage (20 mg/kg body weight/day) for 2 weeks. Mice were fasted overnight, and total RNA was extracted and subjected to quantitative PCR analysis. The relative level of mRNA was measured by comparing it to β-actin. *B*, alterations in MUP-1 mRNA expression during the fasting and refeeding cycle. Total RNA was extracted from the livers of 10-week-old male C57BL/6J mice under a fed status (*fed*), fasted overnight for 18 h (*fasted*), or fasted overnight followed by 1 h of refeeding (*refed*) and was then subjected to real-time PCR analysis to quantify the mRNA abundance of MUP-1 as described in *A*. *, $p < 0.05$; **, $p < 0.01$; $n = 4$–5.

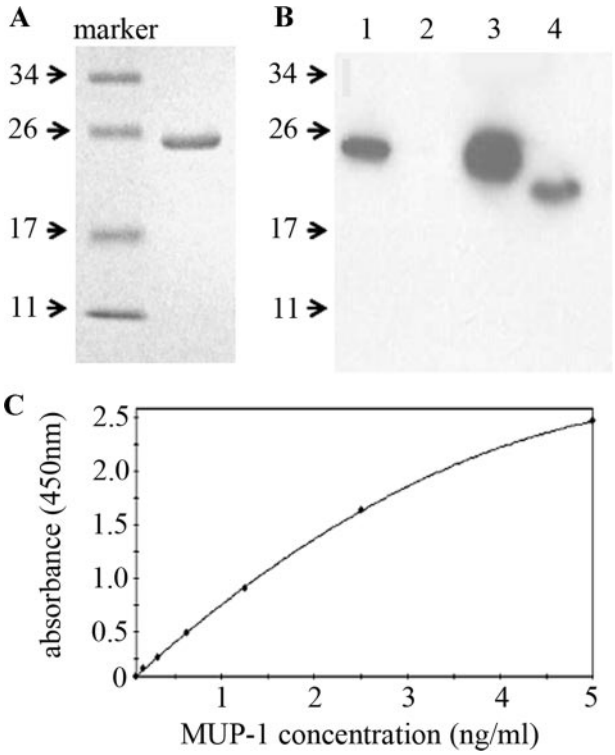

FIGURE 2. **Production of the recombinant MUP-1 protein and anti-MUP-1 antibody and establishment of a sandwich immunoassay for quantification of MUP-1.** *A*, SDS-PAGE analysis of the purified recombinant His_6-tagged MUP-1. The protein samples were separated by 15% polyacrylamide gel under reducing condition and stained with Coomassie Brilliant Blue R250. *B*, Western blot analysis to detect recombinant MUP-1 and endogenous MUP-1 in mouse serum with a rabbit anti-MUP-1 polyclonal antibody. *E. coli* BL21(DE3)-codon plus RILP containing pHtb-MUP-1 plasmid was induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 5 h to express the recombinant MUP-1. *Lane 1*, purified His_6-tagged MUP-1 protein (10 ng); *lane 2*, total cell lysate of *E. coli* before isopropyl 1-thio-β-D-galactopyranoside induction; *lane 3*, total cell lysate of *E. coli* after isopropyl 1-thio-β-D-galactopyranoside induction; *lane 4*, C57 male mouse serum (0.1 μl). *C*, ELISA standard curve generated with different concentrations of recombinant protein as described under "Experimental Procedures."

Circulating Concentrations of MUP-1 Are Markedly Reduced in db/db Diabetic/Obese Mice and High Fat Diet-induced Obese Mice—To investigate whether the changes in mRNA expression of MUP-1 in the liver tissue indeed leads to alterations in its circulating concentrations, we established a traditional ELISA to quantify serum MUP-1 in mice. To this end, we subcloned cDNA encoding the mature mouse MUP-1 (without signal peptide) into a prokaryotic expression vector and expressed this protein with a His_6 tag at the NH_2 terminus. SDS-PAGE analysis showed His_6-tagged recombinant MUP-1 as a single band with a molecular mass of around 23 kDa (Fig. 2*A*). This recombinant protein was then used as the antigen for generation of polyclonal antibodies against MUP-1 in rabbits. Western blot analysis demonstrated that the antibody specifically recognized the recombinant MUP-1 as well as the endogenous MUP-1 in mouse serum as a single band (Fig. 2*B*). A sandwich ELISA was then constructed with this polyclonal antibody, and the assay standard curve generated with the recombinant MUP-1 yielded a consistent r value of >0.985 (Fig. 2*C*). The dynamic range of this assay was between 0.015 and 5 ng/ml.

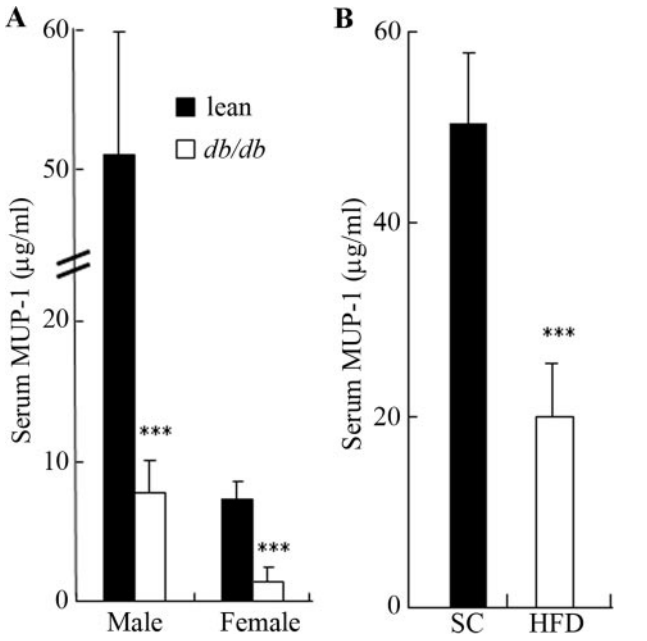

FIGURE 3. **Serum concentrations of MUP-1 protein are decreased in *db*/*db* mice and high fat diet-induced obese mice.** *A*, serum levels of MUP-1 in 12-week-old male and female *db*/*db* mice and their lean littermates. *B*, male C57BL/6J mice were fed with standard chow (*SC*) or high fat diet (*HFD*) for 10 weeks before serum samples were collected. ***, $p < 0.0005$; $n = 4–5$.

Consistent with the changes in MUP-1 mRNA abundance in the liver, serum levels of MUP-1 in male *db*/*db* diabetic mice were markedly reduced compared with the sex- and age-matched lean littermates (51.1 ± 8.8 *versus* 7.8 ± 2.3 μg/ml, $n = 4–5$, $p = 0.00002$) (Fig. 3*A*). Consistent with a previous report (14), we observed a striking sexual dimorphism in MUP-1 expression. Both the mRNA abundance (data not shown) and serum concentration of MUP-1 (Fig. 3*A*) in males was ~7-fold higher than in females. In female mice a similar reduction of MUP-1 in *db*/*db* mice was also observed although to a lesser extent (7.2 ± 1.3 *versus* 1.4 ± 1.0 μg/ml, $n = 10$, $p = 0.0004$).

To further confirm the pathological relevance of the above findings in *db*/*db* mice, we next examined the effects of high fat diet-induced obesity on MUP-1 production in mice. To this end, male C57BL/6J mice were fed with either a standard chow or a high fat diet for a period of 10 weeks. As expected, mice fed with the high fat diet developed obesity, hyperinsulinemia, and insulin resistance (data not shown). Compared with the standard chow group, MUP-1 protein levels in serum were significantly decreased in high fat diet-induced obese mice (50.3 ± 7.5 *versus* 19.8 ± 5.5 μg/ml, $n = 4$, $p = 0.0004$) (Fig. 3*B*).

Replenishment of Recombinant MUP-1 Improves Glucose Intolerance and Insulin Resistance in db/db Mice—The findings described above demonstrate that MUP-1 production is markedly decreased in both dietary obese mice and genetically inherited obese mice with frank diabetes. We next investigated whether supplementation of recombinant MUP-1 had any metabolic effects in these mice. Acute injection of recombinant MUP-1 did not cause any change in the fasting or fed glucose levels, lipid profiles, or serum insulin concentrations in *db*/*db* mice (data not shown). We therefore examined the chronic effects of recombinant MUP-1 in *db*/*db* mice. To this end, mice

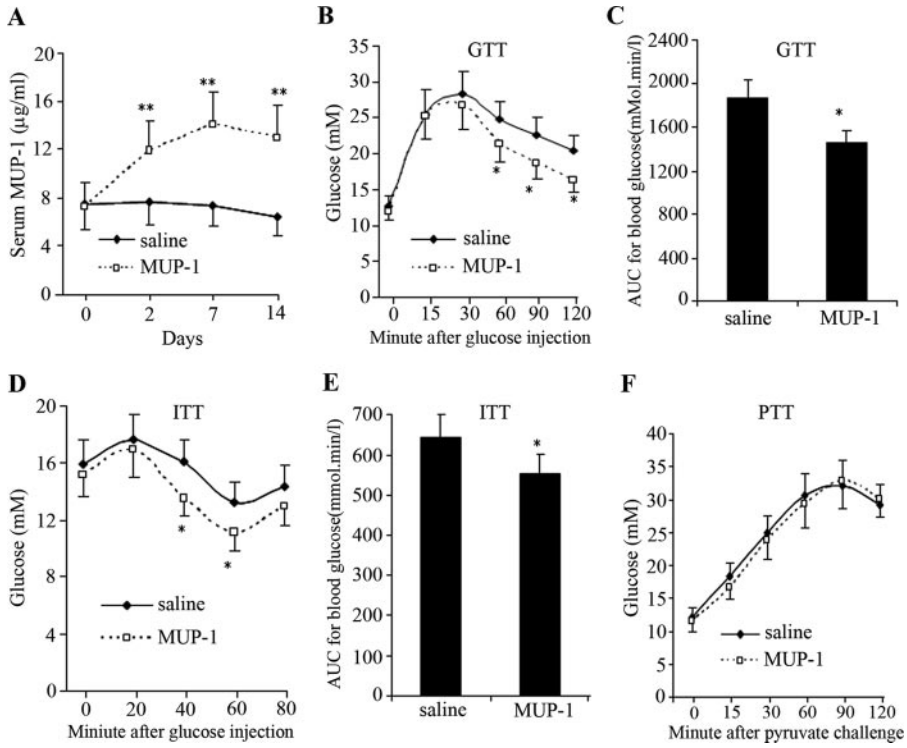

FIGURE 4. **Effects of chronic treatment with recombinant MUP-1 on glucose metabolism and insulin sensitivity in *db*/*db* mice.** 10-week-old male *db*/*db* mice were inserted with Alzet osmotic pumps to deliver recombinant MUP-1 or saline control for a period of 2 weeks as described under "Experimental Procedures." *A*, ELISA measurement of serum MUP-1 protein on various days after treatment. *B*, glucose tolerance test (*GTT*) conducted on day 8 after the treatment. *C*, glucose tolerance test expressed as area under the curve (*AUC*) for blood glucose over 2 h. *D*, insulin tolerance test (*ITT*) conducted on day 10 after the treatment; *E*, insulin tolerance test expressed as area under the curve. *F*, pyruvate tolerance test (*PTT*) on day 12 after the treatment. *, $p < 0.05$; **, $p < 0.01$ *versus* the saline control group; $n = 4–5$.

TABLE 1
Metabolic parameters after chronic treatment with recombinant MUP-1 in *db*/*db* mice

*, $p < 0.05$ *versus* saline control group.

	Saline control[a]	Recombinant MUP-1[a]
Body weight (g)	40.3 ± 2.7	39.1 ± 2.4
Average daily food intake (g)	6.1 ± 0.5	6.3 ± 0.7
Serum insulin (ng/ml)	11.5 ± 1.2	9.2 ± 0.8*
Fasting glucose (mM)	12.8 ± 1.3	11.9 ± 1.0
Fed glucose (mM)	23.7 ± 2.7	19.1 ± 2.1*
Serum triglyceride (mg/dl)	209.3 ± 13.7	176.8 ± 12.4*

[a] $n = 5$.

were surgically implanted with an Alzet osmotic pump, which delivered the recombinant MUP-1 (8 mg/kg of body weight/day) or physiological saline at a constant rate for a period of 2 weeks. Delivery of the recombinant MUP-1 at this dosage caused a ~1.5–2.0-fold elevation of the circulating MUP-1 level throughout the 2-week treatment period (Fig. 4*A*).

Chronic treatment of male *db*/*db* mice with recombinant MUP-1 had no obvious effect on food intake, body weight gains, or fasting blood glucose levels. On the other hand, it caused a significant decrease in the serum levels of triglycerides and insulin (Table 1). The fed glucose levels were also significantly decreased following treatment with recombinant MUP-1. Notably, *db*/*db* mice receiving MUP-1 treatment exhibited significantly improved glucose excursion after glucose load, which was particularly prominent at the late time points of an intraperitoneal glucose tolerance test (Fig. 4*B*). The glucose area under the curve in *db*/*db* mice treated with recombinant MUP-1 was smaller than those treated with saline control (Fig. 4*C*). The insulin tolerance test showed a modest, but significant, increase in insulin sensitivity following chronic treatment with recombinant MUP-1 for 10 days (Fig. 4*D*). On the other hand, a pyruvate tolerance test demonstrated that there was no obvious difference between the two groups in glucose production after pyruvate challenge (Fig. 4*E*). In female *db*/*db* mice, chronic treatment with recombinant MUP-1 also improved glucose tolerance and insulin sensitivity but had no effect on pyruvate-induced glucose production (data not shown).

Chronic Treatment with Recombinant MUP-1 Increases Energy Expenditure and Locomotor Activity in db/db Mice—To further evaluate the metabolic effects of MUP-1 supplementation in *db*/*db* mice, we housed the mice in metabolic cages in order to measure oxygen consumption, respiratory exchange ratio (VCO_2/VCO), and locomotor activity. Replenishment of recombinant MUP-1 significantly elevated energy expenditure in *db*/*db* mice (Fig. 5, *A* and *B*) and caused a modest but significant decrease in the respiratory quotient during the dark cycle (Fig. 5*C*), suggesting that MUP-1 might enhance lipid utilization. Furthermore, supplementation of recombinant MUP-1 also increased the locomotor activity and core body temperature, in particular during the dark cycle (Fig. 5, *D* and *E*). These findings suggest that MUP-1-mediated improvement in glucose tolerance and insulin sensitivity may be attributed in part to an increased energy expenditure and locomotor activity.

Recombinant MUP-1 Selectively Enhances Mitochondrial Biogenesis and Decreases Lipid Accumulation in Skeletal Muscle—To gain insight into the mechanism underlying the actions of MUP-1, we next examined its effects on key metabolic pathways in several major metabolically active tissues including adipose tissue, skeletal muscle, and liver. First, we investigated whether a MUP-1-mediated increase in energy expenditure and an improvement in glucose homeostasis were attributable to elevated mitochondrial uncoupling and heat dissipation in brown adipose tissue. Quantitative real-time PCR analysis revealed that replenishment of recombinant MUP-1 had no obvious effect on the expression of genes involved in either mitochondrial biogenesis or uncoupling, including peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) and uncoupling protein 1 (UCP1), or on the mRNA abundance of a panel of genes encoding mitochondrial proteins

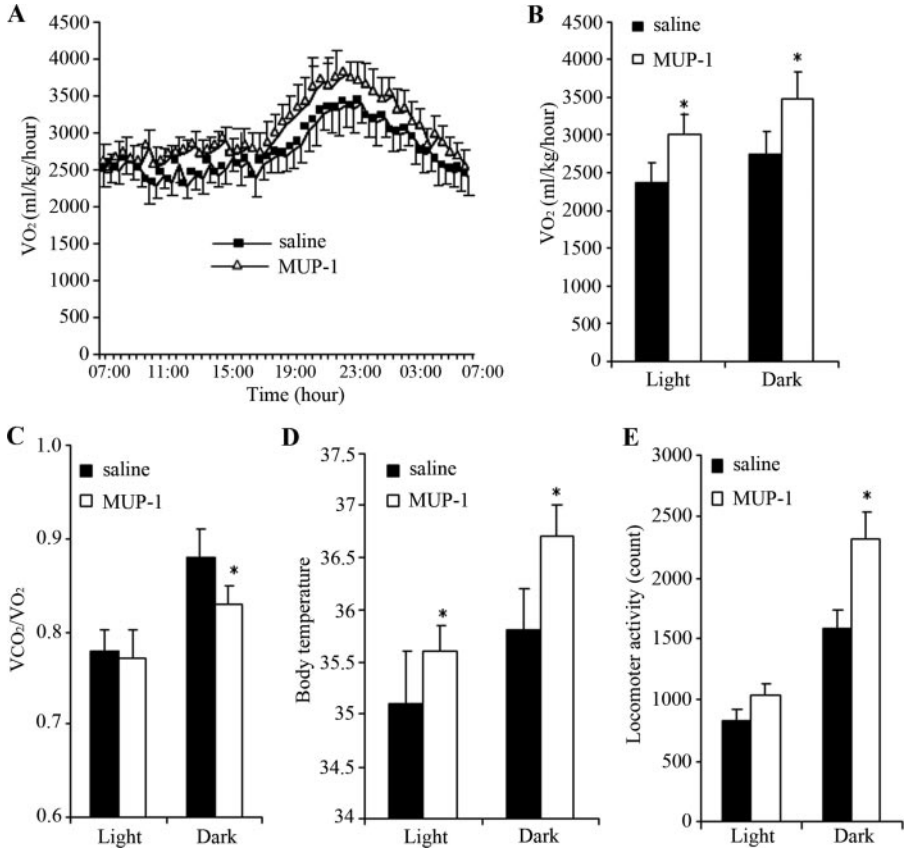

FIGURE 5. **Chronic administration of recombinant MUP-1 enhances energy expenditure, body temperature, and ambulatory activity in *db*/*db* mice.** *A*, oxygen consumption (*VO2*) of *db*/*db* mice treated with saline or recombinant MUP-1 protein. *B*, oxygen consumption expressed as area under the curve during the light and dark phase. *C–E*, bar graphs representing the respiratory quotient, rectal core temperature, and ambulatory activity of the mice, respectively. *, $p < 0.05$; $n = 5–6$.

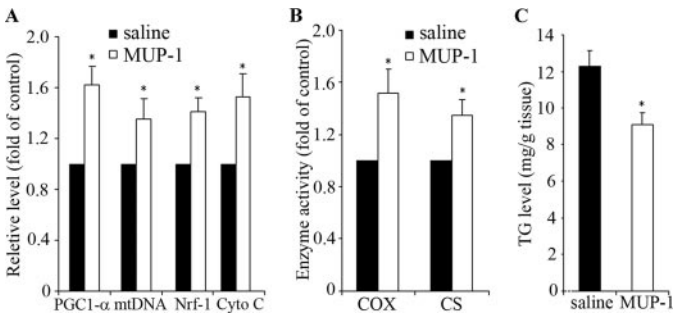

FIGURE 6. **Replenishment of recombinant MUP-1 increases mitochondrial biogenesis and reduces lipid accumulation in skeletal muscle of *db*/*db* mice.** *A*, quantitative real-time PCR analysis of the mRNA abundance of PGC1α, cytochrome *c* (*Cyto C*), and NRF-1 and the mtDNA copy numbers in gastrocnemius muscle. The data are expressed as -fold change relative to saline-treated control. *B*, enzymatic activities of cytochrome *c* oxidase (*COX*) and citrate synthase (*CS*) in gastrocnemius muscle. *C*, triglyceride levels in gastrocnemius muscle as expressed in mg/g of wet tissue. *, $p < 0.05$ *versus* saline control group; $n = 5–6$.

(cytochrome *c*, ATP synthase subunit 6, and NADH dehydrogenase subunit 1).

In contrast to those observations in adipose tissue, we found that replenishment of recombinant MUP-1 significantly increased the expression of several genes involved in mitochondrial biogenesis in skeletal muscle, including PGC1α and nuclear respiratory factor 1 (NRF-1) (Fig. 6*A*). Treatment with recombinant MUP-1 also elevated the mtDNA copy number and gene expression of the mitochondrial protein cytochrome *c* in skeletal muscle. The activities of mitochondrial cytochrome *c* oxidase and citrate synthase, the key components of mitochondrial oxidative phosphorylation and Krebs cycle, respectively, were significantly increased following treatment with recombinant MUP-1 (Fig. 6*B*). Furthermore, this MUP-1-mediated increase in mitochondrial functions was associated with decreased triglyceride content in skeletal muscle (Fig. 6*C*). On the other hand, treatment with recombinant MUP-1 in *db*/*db* mice did not alter the expression of PGC1α and NRF-I, did not affect the mtDNA copy number, and did not change the activities of cytochrome *c* oxidase or citrate synthase in the liver (supplemental Fig. 1, *A* and *B*). In addition, replenishment of recombinant MUP-1 had no obvious effect on triglyceride accumulation and steatosis in the liver as determined by the enzymatic assay and Oil Red O staining (supplemental Fig. 1, *C* and *D*). Taken together, these findings demonstrate that liver-derived MUP-1 preferentially enhances mitochondrial functions and decreases lipid accumulation in skeletal muscle.

Chronic Treatment with Recombinant MUP-1 Increases Insulin-evoked Akt Signaling in Skeletal Muscle—Mitochondrial dysfunction and increased ectopic lipid accumulation have been suggested as important causative factors in insulin resistance (28). We next investigated whether MUP-1-mediated augmentation of mitochondrial activity and reduction in lipid accumulation lead to enhanced insulin signaling in skeletal muscle. To this end, *db*/*db* male mice receiving recombinant MUP-1 or saline control were subjected to a bolus injection of insulin followed by Western blot analysis to quantify phosphorylation of Akt and its downstream target, GSK3β (Fig. 7). This analysis showed that chronic treatment of *db*/*db* mice for a period of 12 days significantly increased phosphorylation of Akt at Ser-473 and GSK3β at Ser-9 in skeletal muscle but not in liver.

DISCUSSION

The members of the MUP family are traditionally thought to be the major protein components in urine, where they modulate behavior by acting as pheromone-binding proteins (17, 29–31). In addition, MUP-1 *per se* has recently been shown to act as a protein pheromone, as recombinant MUP-1 from *E. coli* is capable of promoting male-male aggressive behavior in mice via activation of vomeronasal organ neurons (17). How-

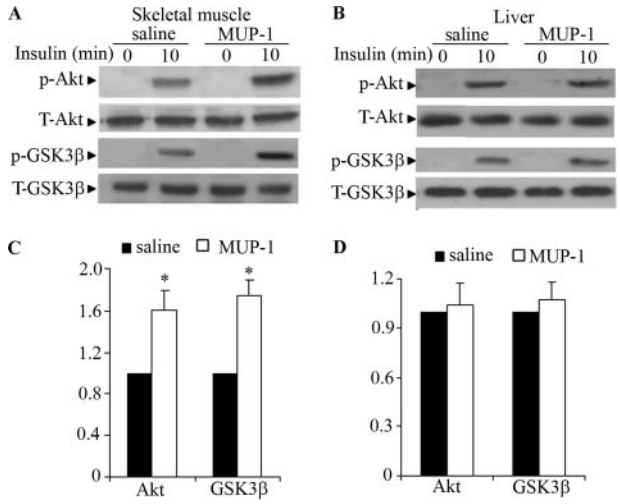

FIGURE 7. **Chronic administration of recombinant MUP-1 increases insulin-evoked Akt signaling in skeletal muscle.** *db*/*db* male mice were treated with recombinant MUP-1 or saline control for 12 days as in Fig. 5 and were then subjected to a bolus injection of insulin. Proteins extracted from gastrocnemius muscle and liver tissues were analyzed by Western blotting. *A* and *C*, representative Western blotting results for total and phosphorylated Akt and GSK3β in gastrocnemius muscle and liver. *C* and *D*, densitometry analysis of phosphorylated Akt and GSK3β in gastrocnemius muscle and liver, respectively. *, $p < 0.05$ *versus* saline control group; $n = 4$.

ever, the relationship between the members of the MUP family and energy metabolism has never been explored thus far. In the present study, we have provided several lines of evidence supporting the role of MUP-1 as a novel metabolic regulator in mice. First, the expression of MUP-1 in the liver is responsive to short term nutritional changes such as fasting and refeeding. In both dietary and genetic obesity, MUP-1 mRNA expression in the liver and its circulating levels are markedly decreased. Second, the PPARγ agonist rosiglitazone increases MUP-1 production, suggesting that elevated MUP-1 is closely related to improved insulin sensitivity. This notion is supported by a recent microarray analysis showing that MUP-1 and its homolog, MUP-3, are among the top-listed genes up-regulated by resveratrol, a polyphenolic compound with insulin-sensitizing activity (32). Third, replenishment of recombinant MUP-1 can partially ameliorate the metabolic defects in *db*/*db* mice, including glucose intolerance and insulin resistance. It is noteworthy that, because of the rapid excretion rate of MUP-1 from the circulatory system to the urine, our osmotic pump-based protein delivery system was only able to partially restore the decreased circulating MUP-1 in *db*/*db* mice (from 8 μg/ml to roughly 15 μg/ml compared with ~50 μg/ml in lean mice). Because of this technical limitation, the metabolic functions of MUP-1 might have been underestimated by using the current approach. It is highly possible that complete restoration of the decreased MUP-1 in *db*/*db* mice could rectify the metabolic disorders to a greater extent. Further study on the transgenic expression of MUP-1 should help to fully elucidate the potential role of MUP-1 in regulating energy metabolism and insulin sensitivity.

Although MUP-1 is produced predominantly in the liver, our data indicate that skeletal muscle, but not the liver, is one of the major metabolic target tissues of MUP-1. This notion is supported by our findings that elevating the plasma levels of MUP-1 by administration of the recombinant protein in *db*/*db* mice preferentially increases mitochondrial biogenesis, decreases the lipid content, and enhances the insulin-evoked Akt signaling cascade in skeletal muscle, whereas these metabolic events are little affected in the liver. Furthermore, the pyruvate tolerance test showed that treatment with recombinant MUP-1 does not change pyruvate-induced glucose production, which occurs predominantly in the liver. Taken together, these findings suggest that MUP-1 might represent one of the long sought after hepatic insulin-sensitizing substances (33), which act in an endocrine manner to exert a beneficial metabolic effect in skeletal muscle.

A growing body of evidence from both animal studies and clinical investigations demonstrate that mitochondrial dysfunction and ectopic lipid accumulation are the important causative factors in the pathogenesis of insulin resistance and type II diabetes (28, 34–36). Lipid metabolites, such as fatty acyl-CoA, diacylglycerol, and ceramide, can impair insulin actions at peripheral tissues by impeding insulin-evoked postreceptor signaling events (37, 38). Because long chain fatty acids are metabolized primarily by mitochondrial β-oxidation, mitochondrial dysfunction has been proposed as a major contributor to impaired lipid oxidation and the accumulation of ectopic fat in skeletal muscle (28, 39, 40). Noticeably, several independent microarray-based studies have shown extensive down-regulation of genes encoding mitochondrial proteins in skeletal muscle from type II diabetic patients as compared with non-diabetic controls (41, 42). In this connection, our present study suggests that the insulin-sensitizing effect of MUP-1 in skeletal muscle is likely to be mediated, at least in part, by its ability to improve mitochondrial functions in mice. This conclusion is supported by our findings that replenishment of recombinant MUP-1 in *db*/*db* mice increased the expression of genes involved in mitochondrial biogenesis and mitochondrial DNA copy number and enhanced the activities of several key enzymes involved in mitochondrial oxidative phosphorylation in skeletal muscle. Of note, a recent study on high fat-induced obese mice showed that an increase in mitochondrial biogenesis, locomoter activity, and energy expenditure mediated by the anti-aging compound resveratrol is also accompanied by a marked elevation of MUP-1 expression in the liver (32). Therefore, the improvement in mitochondrial activities brought about by MUP-1 may also explain the decreased lipid accumulation in skeletal muscle as well as increased energy expenditure and core body temperature in *db*/*db* mice following treatment with recombinant MUP-1.

The detailed mechanism by which MUP-1 enhances mitochondrial functions in skeletal muscle remains to be determined. MUP-1 may exert its metabolic effects via direct activation of an unknown receptor in skeletal muscle or by an indirect mechanism that involves neuromuscular communication. The latter possibility is supported by a recent finding that MUP-1 can activate vomeronasal organ neurons, which characteristically express the G-protein $G\alpha_o$ subunit and Vmn2r putative pheromone receptors (17), which in turn promotes male-male aggressive behavior in mice. Therefore, some of the metabolic effects of MUP-1 are perhaps attributable to its actions on the central nervous system as a pheromone. By stimulation of neu-

ral cells in certain part of the brain, MUP-1 enhances locomotor activity thereby increasing mitochondrial content in skeletal muscle.

In summary, the present study shows for the first time that MUP-1, a secreted protein that belongs structurally to the lipocalin superfamily, is a humoral metabolic regulator in mice. However, unlike in the other members of the lipocalin family, the plasma level of MUP-1 is markedly decreased in both dietary and genetic obesity. Replenishment of recombinant MUP-1 in *db*/*db* mice alleviates insulin resistance and glucose intolerance by improving mitochondrial dysfunction in skeletal muscle, thereby increasing energy expenditure and enhancing insulin signaling. These findings highlight the functional diversity of the lipocalin superfamily and support the role of circulating small lipid-binding proteins as an emerging class of humoral factors that are critically involved in energy metabolism and insulin sensitivity.

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