

Identification of MUP1 as a Regulator for Glucose and Lipid Metabolism in Mice^{*[S]}

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Major urinary protein (MUP) 1 is a lipocalin family member abundantly secreted into the circulation by the liver. MUP1 binds to lipophilic pheromones and is excreted in urine. Urinary MUP1/pheromone complexes mediate chemical communication in rodents. However, it is unclear whether circulatory MUP1 has additional physiological functions. Here we show that MUP1 regulates glucose and lipid metabolism. MUP1 expression was markedly reduced in both genetic and dietary fat-induced obesity and diabetes. Mice were infected with MUP1 adenoviruses via tail vein injection, and recombinant MUP1 was overexpressed in the liver and secreted into the bloodstream. Recombinant MUP1 markedly attenuated hyperglycemia and glucose intolerance in genetic (*db/db*) and dietary fat-induced type 2 diabetic mice as well as in streptozotocin-induced type 1 diabetic mice. MUP1 inhibited the expression of both gluconeogenic genes and lipogenic genes in the liver. Moreover, recombinant MUP1 directly decreased glucose production in primary hepatocyte cultures by inhibiting the expression of gluconeogenic genes. These data suggest that MUP1 regulates systemic glucose and/or lipid metabolism through the paracrine/autocrine regulation of the hepatic gluconeogenic and/or lipogenic programs, respectively.

Circulatory glucose is maintained within a narrow range by a sophisticated regulatory system to provide a constant fuel supply for cell metabolism. The liver plays a key role in the maintenance of systemic glucose homeostasis. In the absorptive state, ingested glucose is taken up by hepatocytes and converted to glycogen and lipids. In the postabsorptive state, hepatocytes produce glucose, which is secreted into the circulation. Insulin and counter-regulatory hormones (e.g. glucagon and glucocorticoids) regulate hepatic glucose production mainly by regulating the hepatic gluconeogenic program. Insulin decreases hepatic glucose production by suppressing the expression of key gluconeogenic genes, including phosphoenolpyruvate carboxykinase (*PEPCK*)² and glucose-6-phosphatase (*G6Pase*);

conversely, counter-regulatory hormones increase hepatic glucose production by stimulating the transcription of these genes. The hepatic gluconeogenic program is activated at abnormally high levels in diabetic subjects, contributing to hyperglycemia (1). Hyperglycemia initiates pathological changes in multiple tissues, contributing to neuropathy, nephropathy, and cardiovascular disorders in both type 1 and type 2 diabetes.

The liver secretes a variety of proteins, including insulin like-growth factor-1 (IGF-1), insulin-like growth factor-binding proteins, fibroblast growth factor-21 (FGF-21) and major urinary proteins (MUPs) (2–6). Insulin-like growth factor-binding proteins and FGF-21 have been well documented to regulate multiple aspects of glucose and lipid metabolism in animals (2, 3, 7). Interestingly, resveratrol treatments markedly increase MUP1 expression (8). Resveratrol also reduces hyperglycemia and improves insulin sensitivity in high fat diet (HFD)-fed mice (8). These observations raise the possibility that MUP1 may be involved in the regulation of glucose and lipid metabolism.

MUP1 is a lipocalin family member containing a characteristic eight-stranded β -barrel structure (5, 9). The mouse *MUP* genes are highly polymorphic, including 20 discrete isoforms (10). Individual mice express 4–6 isoforms of MUPs (11). Different isoforms of MUPs are very similar in DNA sequences (>85% identity). Comparative genomic analyses identify nine *MUP* genes in rats and a single *MUP* gene in other mammals (10). MUP1 (~18 kDa in mass) is abundantly expressed in hepatocytes (3.5–4% of total protein synthesis) and secreted into the bloodstream (6, 12). MUP1 binds via its central hydrophobic pocket to lipophilic molecules, including volatile pheromones (small odorant molecules) (5, 13). The MUP1/pheromone complexes are excreted into the urine through kidney and mediate chemical communication in rodents (5, 14). Individual mice excrete a distinct pattern of different MUP isoforms that signals the identity of the owners (5, 14). MUP1 slowly releases its bound volatile odorant molecules from urinary scent marks into the air, thereby increasing the half-life of pheromones (15). Moreover, MUP themselves also act as pheromone ligands to mediate chemical signaling in mice (10). In this study, we demonstrated that in addition to mediating chemical signaling, circulatory MUP1 also regulates systemic glucose metabolism, presumably by regulating the hepatic gluconeogenic and/or lipogenic programs.

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains a supplemental table.

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² The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; MUP, major urinary protein; hMUP, human MUP; G6Pase, glucose-6-phosphatase; FAS, fatty acid synthase; GTT, glucose tolerance test; ITT, insulin tolerance test; MUP, major urinary protein; β -gal, β -galactosidase; STZ, streptozotocin; SCD1, stearyl-CoA desaturase-1; SREBP, sterol regulatory

element-binding protein; ChREBP, carbohydrate response element binding protein; PPAR γ , peroxisome proliferator-activated receptor- γ ; HFD, high fat diet; qRT-PCR, quantitative real-time RT-PCR; HGP, hepatic glucose production; PGC1 α , proliferator-activated receptor coactivator 1 α ; WT, wild type.

EXPERIMENTAL PROCEDURES

Animals—Wild-type and *db/db* mice were in the C57BL/6 genetic background and purchased from The Jackson Laboratory (Bar Harbor, ME) and housed on a 12-h light and 12-h dark cycle. Mice were fed either normal chow (9% kcal from fat) or a high fat diet (45% kcal from fat, catalog number D12451, Research Diets Inc., New Brunswick, NJ), and had a free access to water. All animal experiments were conducted in accordance with the protocols approved by the University Committee on Use and Care of Animals (UCUCA).

MUP1 Adenoviral Preparation and Purification—Total RNA was extracted from mouse liver using TRIzol reagent (Invitrogen) and used to synthesize the first strand cDNAs by reverse transcription using oligo(dT)_{12–18} and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Mouse MUP1 cDNA was generated by PCR using these hepatic cDNAs and verified by DNA sequencing. Full-length MUP1 cDNA was used to generate recombinant MUP1 adenoviruses using a pAdEasy kit (QBiogene, Carlsbad, CA). Recombinant adenoviruses were amplified in QBI-293A cells and purified by CsCl gradient ultracentrifugation.

Blood Glucose and Insulin—Blood samples were collected from mouse tail veins using heparin-pretreated capillary tubes. Blood glucose was measured by a glucometer (Bayer Corp., Tarrytown, NY), and plasma insulin was measured using a rat insulin enzyme-linked immunosorbent assay kit (Crystal Chem, Inc., Chicago, IL).

Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)—For GTT, mice were fasted overnight and intraperitoneally injected with D-glucose. Blood glucose was measured 0, 15, 30, 60, and 120 min after glucose injection. For ITT, mice were fasted for 6 h (from 10:00 a.m. to 4:00 p.m.) and intraperitoneally injected with human insulin. Blood glucose was monitored 0, 15, 30, and 60 min after insulin injection.

Streptozotocin (STZ) Treatment and Adenoviral Infection—STZ (Sigma) was dissolved in 0.1 M sodium citrate (pH 4.5) immediately before use. Wild-type male mice (10 weeks) were intraperitoneally injected with STZ (200 mg/kg of body weight). Blood glucose was measured in randomly fed mice 3 days after STZ injection. Additional STZ (100 mg/kg of body weight) was injected into a few mice whose blood glucose levels were below 300 mg/dl. Type 1 diabetes was defined by hyperglycemia (blood glucose levels were >300 mg/dl in randomly fed mice). MUP1 or β -galactosidase (β -gal) (control) adenoviruses were administered into diabetic mice via tail vein injection at 2×10^{11} viral particles per mouse.

Hepatic Glycogen Contents—Liver tissues were boiled in 30% KOH. Glycogen was precipitated with ethanol and air-dried. Glycogen pellet was dissolved in water and digested with amyloglucosidase (Sigma) at 42 °C. Glycogen-derived glucose was measured using a glucose assay kit (Stanbio Laboratory, Boerne, TX).

Hepatic Triglyceride Contents—Liver tissues were homogenized in chloroform:methanol (2:1) and separated by centrifugation. The organic phase was air-dried. Triglycerides was dissolved and hydrolyzed in 3 M KOH at 70 °C. Glycerol

concentration was measured using a glycerol assay kit (Sigma) and converted to triglyceride concentration.

Quantitative Real-time RT-PCR (qRT-PCR)—Mice were fasted overnight and euthanized at 10 am. Liver was isolated immediately and flash-frozen in liquid nitrogen. Total RNA was extracted from the liver and used to synthesize the first strand cDNAs by reverse transcription using oligo(dT)_{12–18} and Moloney murine leukemia virus reverse transcriptase. The mRNA abundance of various molecules was measured using the Brilliant SYBR Green QPCR kit and the Mx3000P real-time PCR system (Stratagene, La Jolla, CA) as we previously described (16). qRT-PCR primer sequences are listed in supplemental Table 1.

Immunoprecipitation and Immunoblotting—Tissues were homogenized in lysis buffer (50 mM Tris, pH 7.5; 1% Nonidet P-40; 150 mM NaCl; 2 mM EGTA; 1 mM Na₃VO₄; 100 mM NaF; 10 mM Na₄P₂O₇; 1 mM benzamide; 10 μ g/ml aprotinin; 10 μ g/ml leupeptin; 1 mM phenylmethylsulfonyl fluoride). Protein concentration in tissue lysate was measured using a Bio-Rad protein assay kit (Bio-Rad). Tissue extracts were incubated with antibodies at 4 °C for 2 h and with protein A-agarose beads (RepliGen Corp., Waltham, MA) for an additional hour at 4 °C. The immunocomplexes absorbed on the protein A-agarose beads were washed three times with washing buffer (50 mM Tris, pH 7.5; 1% Nonidet P-40; 150 mM NaCl; 2 mM EGTA) and boiled at 95 °C for 5 min in loading buffer (50 mM Tris-HCl, pH 6.8; 2% SDS, 2% β -mercaptoethanol; 10% glycerol; 0.005% bromophenol blue). Protein was separated by SDS-PAGE, electro-transferred onto nitrocellulose membranes, immunoblotted with indicated antibodies, and visualized by the Odyssey infrared imaging system (Li-COR, Lincoln, NE).

Primary Hepatocyte Culture and the Gluconeogenic Assays—Hepatocytes were isolated from male mice (9 weeks in C57BL/6 background). Briefly, mice were anesthetized, and the liver was perfused with 0.5 mg/ml type II collagenase (Worthington Biochemical Co.) via the inferior vena cava to isolate hepatocytes. Cells were seeded for 3 h on collagen-coated 12-well plates in Williams Medium E (catalog number: W4125, Sigma) supplemented with 2% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin ($\sim 4 \times 10^5$ cells/well). For viral infection experiments, cells were incubated with β -gal or MUP1 adenoviruses (2×10^9 viral particles/well) for 2 h, cultured overnight (~ 16 h) in Williams Medium E supplemented with 1% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin and then subjected to hepatic glucose production (HGP) assays. For conditioned medium treatment experiments, hepatocytes were incubated with β -gal (control) or MUP1 conditioned medium overnight and then subjected to HGP assays. To prepare conditioned medium, HepG2 cells were infected with β -gal or MUP1 adenoviruses for 4 h and grown overnight in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. The cells were subsequently grown in Williams Medium E supplemented with 2% fetal bovine serum, and culture medium (conditioned medium) was collected 24 h later. For HGP assays, primary hepatocyte cultures were washed twice with phosphate-buffered saline and incubated with HGP buffer (phenol red- and glucose-free Dulbecco's modified Eagle's medium supplemented with 0.8%

MUP1 Reduction of Blood Glucose

bovine serum albumin, 10 mM sodium DL-lactate) in the presence or absence of MIX (10 μ M *N*⁶,2'-*O*-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt and 100 nM dexamethasone). Culture buffer was collected 4 h later and used to measure glucose using Glucose LiquiColor kits (Fisher Scientific Inc.).

Statistical Analysis—Data were presented as means \pm standard error of the mean (S.E.). Two-tailed Student's *t* tests were used for comparisons between two groups. Statistical significance was considered at *p* < 0.05.

RESULTS

Obesity and Type 2 Diabetes Are Associated with Reduced MUP1 Expression—To determine whether MUP1 expression is regulated by metabolism, male mice (15–16 weeks) were either fasted overnight or refeed for 4 h after fasting. Total hepatic RNA was prepared and used to measure the expression of MUP1 and key gluconeogenic genes by real-time qRT-PCR. Refeeding significantly reduced the expression of *PEPCK* and *PGC1 α* , two important gluconeogenic genes (Fig. 1A). Interestingly, refeeding also reduced MUP1 expression by 72% (Fig. 1A). These results suggest that MUP1 expression is regulated by metabolic signals.

Metabolism is profoundly changed in obesity and type 2 diabetes. To determine whether MUP1 expression is altered in obesity and type 2 diabetes, wild-type and *db/db* mice (10–11 weeks) were fasted overnight. Total hepatic RNA was prepared and used to measure hepatic MUP1 expression by qRT-PCR. *db/db* mice are deficient of functional leptin receptor and commonly used as a genetic model of obesity and type 2 diabetes. *db/db* mice were severely obese (body weight: *db/db*, 40.8 \pm 1.6 g, *n* = 5; wild type (WT), 20.0 \pm 0.3 g, *n* = 5, *p* < 0.05) and hyperglycemic (fasting blood glucose: *db/db*, 191.8 \pm 19.5 mg/dl, *n* = 5; WT, 70.8 \pm 1.7 mg/dl, *n* = 5, *p* < 0.05). Importantly, MUP1 expression was reduced by \sim 90% in *db/db* mice (Fig. 1B).

To determine whether dietary fat-induced obesity and type 2 diabetes are also associated with a reduction in MUP1 expression, wild-type male mice (8 weeks) were fed an HFD for 8 weeks. As expected, HFD-fed mice developed both obesity (normal chow: 24.0 \pm 0.4 g, *n* = 8; HFD: 35.7 \pm 1.1 g, *n* = 9, *p* < 0.05) and hyperglycemia (fasting blood glucose: normal chow: 80.4 \pm 5.9 mg/dl, *n* = 8; HFD: 136.7 \pm 8.9 mg/dl, *n* = 9, *p* < 0.05). MUP1 expression was decreased by \sim 68% in HFD-fed mice (Fig. 1C).

To determine whether MUP1 protein is also reduced in diabetic mice, liver extracts were prepared from wild-type and *db/db* mice and immunoblotted with anti-MUP1 antibody (α MUP1) or α -tubulin, respectively. Two forms of MUP1 were detected in wild-type mice (Fig. 1D). These two forms may be generated through differential proteolytic cleavages of the MUP1 precursors. Importantly, MUP1 protein levels were markedly reduced in *db/db* mice, and the low molecular weight form was decreased to a greater extent (Fig. 1D). Moreover, both the high and the low molecular weight forms of MUP1 were also markedly reduced in livers of HFD-fed mice (Fig. 1D). These observations raise the possibility that a reduction in

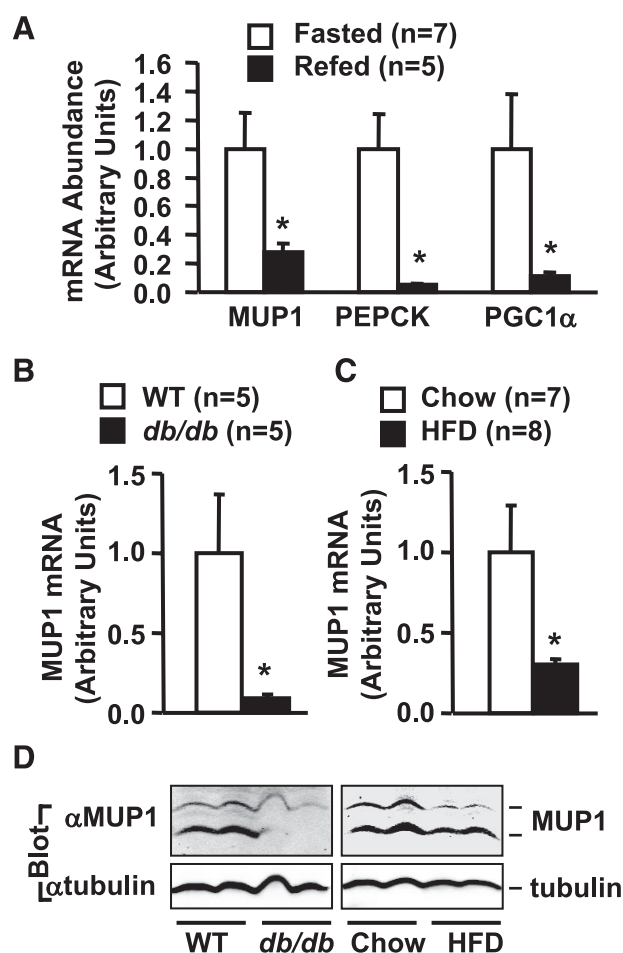


FIGURE 1. A reduction of hepatic MUP1 expression is associated with obesity and type 2 diabetes. A, male mice (15–16 weeks) were fasted overnight or refeed for 4 h after overnight fasting. Total RNA was prepared from the liver and used to measure MUP1, PEPCK, PGC1 α , and β -actin mRNA abundance by qRT-PCR. MUP1, PEPCK, and PGC1 α expressions were normalized to β -actin expression. B, WT and *db/db* males (10–11 weeks) were fasted overnight. Total hepatic RNA was prepared and used to measure MUP1 mRNA abundance by qRT-PCR. MUP1 expression was normalized to β -actin expression. C, male mice (8 weeks) were fed an HFD for 8 weeks and fasted overnight. MUP1 expression was measured by qRT-PCR. D, liver extracts were prepared from WT or *db/db* male mice (16 weeks) and immunoblotted with anti-MUP1 (α MUP1) or α -tubulin. In parallel experiments, WT mice (7 weeks) were fed normal chow or HFD for 10–11 weeks. Liver extracts were immunoblotted with α MUP1 or α -tubulin. *, *p* < 0.05. Error bars indicate S.E.

MUP1 expression may contribute to hyperglycemia in obese and diabetic animals.

MUP1 Ameliorates Hyperglycemia and Glucose Intolerance in Diabetic Mice—To determine whether MUP1 is involved in the regulation of glucose metabolism, MUP1 cDNA was prepared from mouse livers and used to generate recombinant MUP1 adenoviruses. To determine whether MUP1 adenoviruses express recombinant MUP1, HepG2 cells, which are derived from human livers, were infected with β -gal or MUP1 adenoviruses. Cell extracts were prepared 48 h after viral infection and immunoblotted with anti-MUP1 (α MUP1) or anti- β -actin antibodies. MUP1 protein was detected in MUP1 but not β -gal adenovirus-infected cells (Fig. 2A). To determine whether recombinant MUP1 is a secretory protein, culture medium was prepared from HepG2 cells 48 h after viral infection and immunoblotted with α MUP1. Recombinant MUP1 was detected in

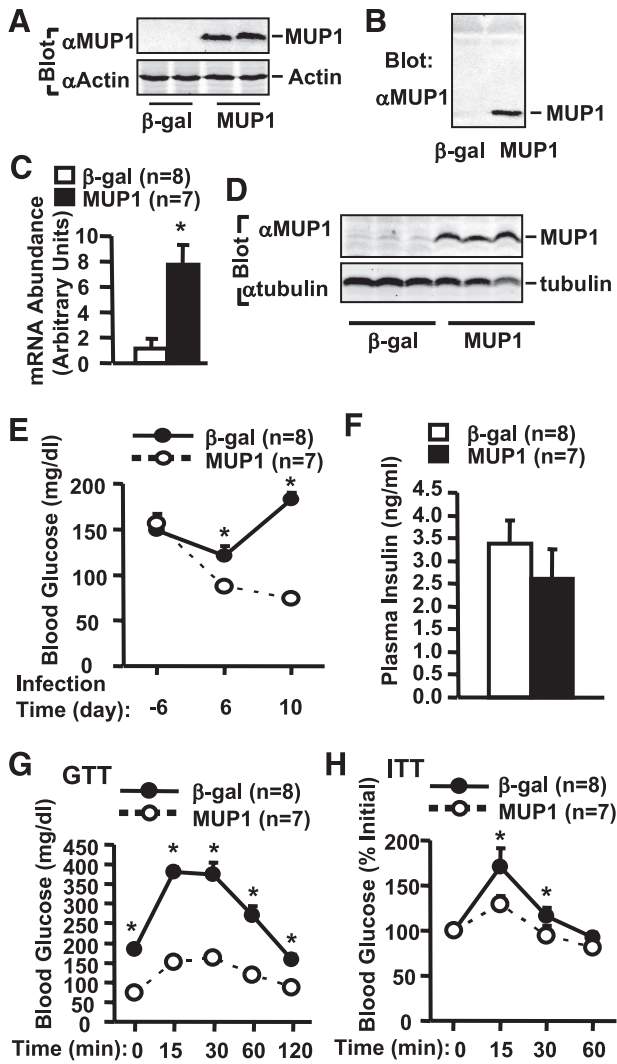


FIGURE 2. MUP1 improves hyperglycemia and glucose intolerance in genetic type 2 diabetes. *A*, HepG2 cells were infected with β -gal or MUP1 adenoviruses. Cell extracts were prepared 48 h after infection and immunoblotted with α MUP1 or anti- β -actin. *B*, HepG2 cells were infected with β -gal or MUP1 adenoviruses. Culture medium was changed 24 h after infection, collected 24 h later, and immunoblotted with α MUP1. *C*, *db/db* male mice (9 weeks) were infected with β -gal or MUP1 adenoviruses via tail vein injection. Mice (16 days after infection) were fasted overnight, and livers were harvested. Total hepatic RNA was prepared and used to measure MUP1 mRNA by qRT-PCR. MUP1 expression was normalized to β -actin expression. *D*, male mice (7 weeks) were fed HFD for 10 weeks and infected with β -gal or MUP1 adenoviruses via tail vein injection. Liver extracts were prepared 16 days after viral infection and immunoblotted with α MUP1 or α -tubulin as indicated. *E–H*, *db/db* male mice (9 weeks) were infected with β -gal or MUP1 adenoviruses via tail vein injection. *E*, fasting blood glucose (–6, 6 days prior to viral infection). *F*, fasting plasma insulin (6 days after viral infection). *G*, GTT. Mice (10 days after viral infection) were fasted overnight and intraperitoneally injected with D-glucose (0.8 g/kg of body weight). Blood glucose was monitored 0, 15, 30, 60, and 120 min after injection. *H*, ITT. Mice (7 days after infection) were fasted for 6 h and intraperitoneally injected with human insulin (2.5 units/kg of body weight). Blood glucose was measured 0, 15, 30, and 60 min after injection. *, $p < 0.05$. Error bars indicate S.E.

the medium collected from MUP1 but not β -gal adenovirus-infected HepG2 cells (Fig. 2*B*).

To determine whether recombinant MUP1 is also expressed in MUP1 adenovirus-infected mice, *db/db* male mice (9 weeks) were infected with either MUP1 or β -gal adenoviruses via tail vein injection. Recombinant adenoviruses primarily infect hepatocytes under these conditions (17). Total liver RNA was

prepared 16 days after viral infection and used to measure MUP1 mRNA abundance by qRT-PCR. MUP1 expression was approximately six times higher in MUP1 adenovirus-infected mice than in β -gal adenovirus-infected mice (Fig. 2*C*). To verify recombinant MUP1 proteins, mice were infected with β -gal or MUP1 adenoviruses. Liver extracts were prepared 16 days after infection and immunoblotted with α MUP1 or α -tubulin. MUP1 protein levels were markedly increased in livers of MUP1 adenovirus-infected mice (Fig. 2*D*). Together, these data indicate that recombinant MUP1 is highly expressed in livers of MUP1 but not β -gal adenovirus-infected mice and secreted into the circulation.

To determine whether recombinant MUP1 regulates blood glucose in animals, *db/db* male mice (9 weeks) were infected with MUP1 or β -gal adenoviruses, respectively. Blood glucose was measured in animals fasted overnight. Blood glucose was similar between these two groups prior to viral infection (Fig. 2*E*, –6 days). Fasting blood glucose slightly decreased 6 days after β -gal adenoviral infection and increased 10 days after β -gal adenoviral infection (Fig. 2*E*). In contrast, blood glucose decreased to a much greater extent in MUP1 adenovirus-infected mice than in β -gal adenovirus-infected mice 10 days after infection. Blood glucose was 59.4% lower in MUP1 adenovirus-infected mice than in β -gal adenovirus-infected mice 10 days after infection. MUP1 overexpression slightly reduced blood insulin in *db/db* mice; however, the values did not reach statistical difference between MUP1 and β -gal adenovirus-infected mice (Fig. 2*F*). MUP1 overexpression did not alter body weight under these conditions (β -gal: 41.5 ± 0.9 g, $n = 8$; MUP1: 39.2 ± 1.2 g, $n = 7$, $p = 0.16$; 7 days after viral infection).

To further analyze the effect of MUP1 on glucose metabolism, GTT and ITT were performed in *db/db* mice 10 and 7 days after adenoviral infection, respectively. Blood glucose levels were much lower in MUP1 than in β -gal adenovirus-infected mice at each time point in GTT (Fig. 2*G*). Exogenous insulin reduced blood glucose to a greater extent in MUP1 than in β -gal adenovirus-infected mice 15 and 30 min after injection in ITT (Fig. 2*H*). Together, these data demonstrate that recombinant MUP1 markedly attenuates hyperglycemia and glucose intolerance in genetically diabetic mice.

MUP1 Overexpression Results in the Inhibition of the Hepatic Gluconeogenic Program in *db/db* Mice—To determine whether MUP1 ameliorates hyperglycemia by decreasing endogenous glucose production in the liver, we examined the hepatic gluconeogenic program by measuring the expression of *PEPCK*, *G6Pase*, and *PGC1 α* , key gluconeogenic genes. *db/db* male mice (9 weeks) were infected with MUP1 or β -gal adenoviruses, respectively. Sixteen days after viral infection, mice were fasted overnight, and livers were isolated. The mRNA abundance of hepatic *PEPCK*, *G6Pase*, and *PGC1 α* was measured by qRT-PCR and normalized to β -actin mRNA levels. MUP1 inhibited the expression of *PEPCK* by 32% and *G6Pase* by 44% (Fig. 3*A*). In contrast, *PGC1 α* expression was similar between MUP1 and β -gal adenovirus-infected mice (Fig. 3*A*).

To determine whether overexpression of MUP1 alters insulin signaling, we measured the phosphorylation of the insulin receptor and Akt. Akt is a key downstream mediator of insulin action (1, 18–20). *db/db* male mice (9 weeks) were infected

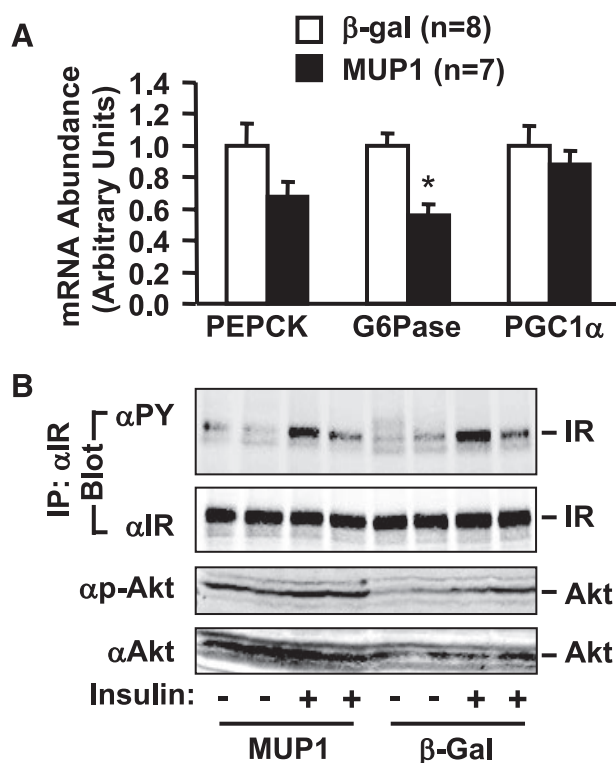


FIGURE 3. MUP1 inhibits G6Pase expression but not insulin signaling in the liver. *db/db* male mice (9 weeks) were infected with β -gal or MUP1 adenoviruses via tail vein injection. **A**, mice (16 days after infection) were fasted overnight, and livers were harvested. Total hepatic RNA was prepared and used to measure the abundance of PEPCK, G6Pase, and β -actin mRNAs by qRT-PCR. PEPCK or G6Pase expression was normalized to β -actin expression. **B**, mice (16 days after infection) were fasted overnight and injected with human insulin (three units per mouse) via the inferior vena cava. Liver extracts were prepared 5 min after insulin injection, immunoprecipitated (IP) with anti-insulin receptor antibody (α IR), and immunoblotted with anti-phosphotyrosine antibody (α PY). The same blots were reprobed with anti-insulin receptor antibody. Tissue extracts were immunoblotted with anti-phospho-Akt (p-Thr³⁰⁸) (α p-Akt), and the same blots were reprobed with α Akt. *, $p < 0.05$. Error bars indicate S.E.

with MUP1 or β -gal adenoviruses. Mice were fasted overnight 16 days after viral infection and treated with insulin for 5 min. Liver extracts were immunoprecipitated with anti-insulin receptor antibody (Fig. 3B, α IR) and immunoblotted with anti-phosphotyrosine antibody (Fig. 3B, α PY). Insulin similarly stimulated tyrosine phosphorylation of the insulin receptor in both MUP1 and β -gal adenovirus-infected mice (Fig. 3B). Insulin-stimulated phosphorylation of Akt (pThr³⁰⁸) was also comparable between MUP1 and β -gal adenovirus-infected mice (Fig. 3B). Therefore, it is unlikely that MUP1 inhibits the hepatic gluconeogenic program by increasing insulin sensitivity in the liver.

MUP1 Inhibits the Hepatic Lipogenic Program in *db/db* Mice—To determine whether hepatic overexpression of MUP1 alters lipid metabolism in the liver, *db/db* male mice (9 weeks) were infected with MUP1 or β -gal adenoviruses for 16 days. Mice were fasted overnight, and livers were isolated. Liver weight was significantly reduced in MUP1 adenovirus-infected mice (Fig. 4A). Hepatic glycogen content was also reduced slightly but not significantly (Fig. 4B). Interestingly, hepatic triglyceride contents were reduced by 53% in MUP1 adenovirus-infected mice

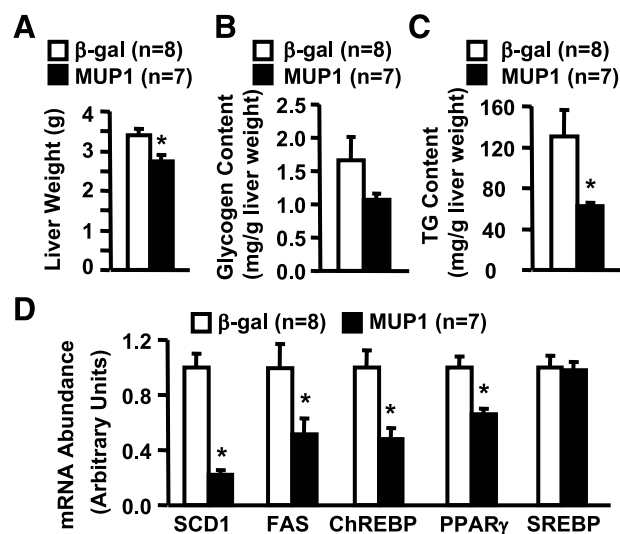


FIGURE 4. MUP1 inhibits the hepatic lipogenic program in *db/db* mice. *db/db* male mice (9 weeks) were infected with β -gal or MUP1 adenoviruses via tail vein injection. Sixteen days after viral infection, mice were fasted overnight and sacrificed. **A**, liver weight. **B**, hepatic glycogen content. **C**, hepatic triglyceride (TG) content. **D**, total hepatic RNA was prepared and used to measure the mRNA abundance of SCD1, FAS, ChREBP, PPAR γ , and SREBP by qRT-PCR. The expression of SCD1, FAS, ChREBP, PPAR γ , and SREBP was normalized to β -actin expression. Error bars indicate S.E.

when compared with that in β -gal adenovirus-infected mice (Fig. 4C).

To determine whether MUP1 regulates the hepatic lipogenic program in *db/db* mice, the expression of lipogenic genes was measured by qRT-PCR. Hepatic overexpression of MUP1 suppressed the expression of stearoyl-CoA desaturase-1 (SCD1) by 78%, fatty acid synthase (FAS) by 51%, carbohydrate response element binding protein (ChREBP) by 52%, and peroxisome proliferator-activated receptor- γ (PPAR γ) by 34% (Fig. 4D). In contrast, SREBP1c expression was similar between MUP1 and β -gal adenovirus-infected mice (Fig. 4D). These data suggest that MUP1 inhibits the hepatic lipogenesis by suppressing the expression of lipogenic genes, including *SCD1*, *FAS*, *ChREBP*, and *PPAR γ* .

MUP1 Improves Glucose Metabolism in Mice Fed HFD—To determine whether MUP1 ameliorates hyperglycemia and glucose intolerance in dietary fat-induced type 2 diabetes, male mice (7 weeks) were fed HFD for 10 weeks. HFD induced obesity, hyperglycemia, and insulin resistance as described before. Mice were infected with MUP1 or β -gal adenoviruses, and blood glucose, ITT, and GTT were measured 6, 8, and 10 days after viral infection, respectively. Fasting blood glucose was significantly lower in MUP1 adenovirus-infected mice than in β -gal adenovirus-infected mice (Fig. 5A). Blood glucose was also significantly lower in MUP1 adenovirus-infected mice than in β -gal adenovirus-infected mice at each time point in ITT and 0, 15, and 30 min after glucose injection in GTT (Fig. 5, B and C). MUP1 did not alter body weight under these conditions (β -gal: 36.9 \pm 0.9 g, $n = 8$; MUP1: 35.0 \pm 1.3 g, $n = 9$, $p = 0.24$; 7 days after viral infection). These data indicate that MUP1 improves glucose metabolism in both genetic and dietary fat-induced type 2 diabetes.

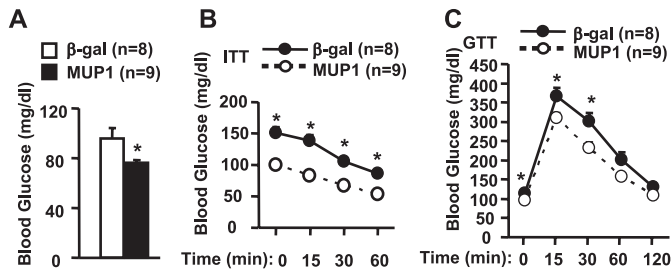


FIGURE 5. MUP1 ameliorates dietary fat-induced hyperglycemia and glucose intolerance. Male mice (7 weeks) were fed HFD for 10 weeks and infected with β -gal or MUP1 adenoviruses via tail vein injection. *A*, fasting blood glucose 6 days after viral infection. *B*, ITT. Mice (8 days after infection) were fasted for 6 h and intraperitoneally injected with human insulin (1 unit/kg of body weight). Blood glucose was monitored 0, 15, 30, and 60 min after injection. *C*, GTT. Mice (10 days after viral infection) were fasted overnight and intraperitoneally injected with D-glucose (2 g/kg of body weight). Blood glucose was monitored 0, 15, 30, 60, and 120 min after injection. *, $p < 0.05$. Error bars indicate S.E.

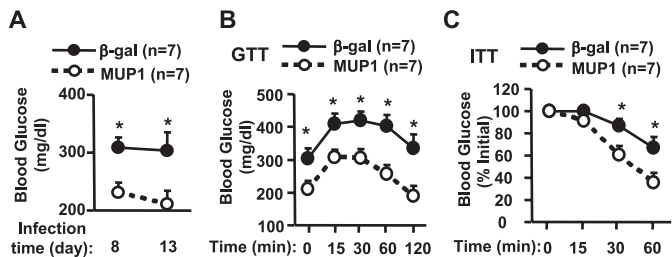


FIGURE 6. MUP1 attenuates hyperglycemia and glucose intolerance in streptozotocin-induced type 1 diabetes. Male mice (10 weeks) were intraperitoneally injected with streptozotocin (200 mg/kg of body weight) to induce type 1 diabetes (randomly fed blood glucose: >300 mg/dl). Diabetic mice were infected with β -gal or MUP1 adenoviruses via tail vein injection. *A*, fasting blood glucose. *B*, GTT (0.3 g/kg of body weight) was conducted on mice 13 days after viral infection. *C*, ITT (1 unit/kg of body weight) was conducted on mice 11 days after viral infection. *, $p < 0.05$. Error bars indicate S.E.

MUP1 Reduces Hyperglycemia and Glucose Intolerance in Streptozotocin-induced Type 1 Diabetes—To determine whether insulin is required for MUP1 to reduce blood glucose, male mice (10 weeks) were administered with STZ, a pancreatic β cell toxin. STZ treatments induced type 1 diabetes as revealed by severe hyperglycemia (blood glucose in randomly fed mice: 457.0 ± 24.5 mg/dl, $n = 14$). STZ-treated mice were infected with either MUP1 or β -gal adenoviruses, respectively. Blood glucose levels in randomly fed mice were similar between these two groups prior to viral infection (MUP1: 459.6 ± 40.6 mg/dl, $n = 7$; β -gal: 454.4 ± 30.9 mg/dl, $n = 7$). Importantly, blood glucose was 25% lower in MUP1 adenovirus-infected mice than in β -gal adenovirus-infected mice 8 days after viral infection and 31% lower in MUP1 than in β -gal adenovirus-infected mice 13 days after viral infection (Fig. 6*A*). In GTT, blood glucose levels were significantly lower in MUP1 adenovirus-infected mice than in β -gal adenovirus-infected mice at each time point after glucose injection (Fig. 6*B*). Exogenous insulin reduced blood glucose to a greater extent in MUP1 adenovirus-infected mice than in β -gal adenovirus-infected mice 30 and 60 min after injection (Fig. 6*C*). Because hyperglycemia induces insulin resistance, MUP1 may improve insulin sensitivity partially by reducing hyperglycemia in STZ-treated mice. Together, these data suggest that MUP1 is able to reduce hyperglycemia and glucose intolerance independent of insulin in type 1 diabetes.

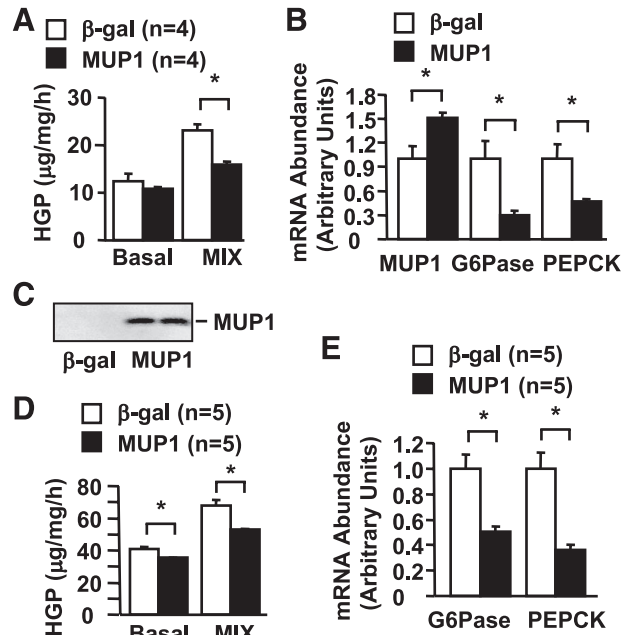


FIGURE 7. MUP1 directly inhibits the gluconeogenic program. *A*, primary hepatocyte cultures were infected with β -gal or MUP1 adenoviruses and treated for 4 h with or without $10 \mu\text{M}$ $N^6,2'$ - O -dibutyryladenosine 3',5'-cyclic monophosphate sodium salt and 100 nM dexamethasone (MIX). HGP was measured and normalized to total protein levels. *B*, primary hepatocyte cultures were infected with β -gal or MUP1 adenoviruses and treated with MIX for 4 h. MUP1, G6Pase, and PEPCK mRNA abundance was measured by qRT-PCR and normalized to β -actin expression. *C*, β -gal or MUP1 conditioned medium was prepared from β -gal or MUP1 adenovirus-infected HepG2 cells and immunoblotted with α MUP1. *D*, primary hepatocyte cultures were treated with β -gal or MUP1 conditioned medium and subjected to HGP assays in the presence or absence of MIX. *E*, primary hepatocyte cultures were treated with in β -gal or MUP1 conditioned medium overnight and then with MIX for additional 4 h. G6Pase and PEPCK mRNA abundance was measured by qRT-PCR and normalized to β -actin expression. *, $p < 0.05$. Error bars indicate S.E.

MUP1 Directly Inhibits the Hepatic Gluconeogenic Program in Primary Hepatocyte Cultures—To determine whether MUP1 directly regulates hepatocyte activity, primary hepatocyte cultures were prepared from male mice (9 weeks), infected with β -gal or MUP1 adenoviruses, treated with or without MIX (a combination of $N^6,2'$ - O -dibutyryladenosine 3',5'-cyclic monophosphate sodium salt, a cAMP analog, and dexamethasone), and then subjected to HGP assays. MIX was used to mimic counter-regulatory hormones to stimulate HGP. MUP1 slightly reduced HGP under basal conditions; however, it reduced HGP by 31% in MIX-stimulated hepatocytes (Fig. 7*A*). To examine the hepatic gluconeogenic program, the expression of key gluconeogenic genes was measured by qRT-PCR. MUP1 expression was higher in MUP1 adenovirus-infected cells than in β -gal adenovirus-infected cells as expected. Importantly, MUP1 overexpression inhibited G6Pase and PEPCK expression by 70 and 54%, respectively (Fig. 7*B*).

To determine whether recombinant MUP1 protein directly regulates the hepatic gluconeogenic program, conditioned medium was prepared from β -gal or MUP1 adenovirus-infected HepG2 cells. Recombinant MUP1 protein was detected in MUP1 but not β -gal conditioned medium (Fig. 7*C*). Hepatocytes were treated with β -gal or MUP1 conditioned medium overnight and then stimulated with or without MIX for 4 h. When compared with β -gal conditioned medium, MUP1 con-

MUP1 Reduction of Blood Glucose

ditioned medium reduced basal and MIX-stimulated HGP by 13 and 22%, respectively (Fig. 7D). In MIX-treated hepatocytes, recombinant MUP1 inhibited the expression of G6Pase by 50% and PEPCK by 64% (Fig. 7E). Taken together, these data suggest that MUP1 directly inhibits the hepatic gluconeogenic program, thereby regulating systemic glucose metabolism.

DISCUSSION

In this study, we discovered that in addition to mediating chemical signaling, circulatory MUP1 also regulates glucose and lipid metabolism in animals. First, we showed that the expression of hepatic MUP1 was regulated by nutritional and/or metabolic signals. Second, the expression of hepatic MUP1 markedly decreased in both genetic and dietary fat-induced type 2 diabetes, suggesting that a reduction of MUP1 contributes to hyperglycemia, insulin resistance, and/or glucose intolerance in diabetes. Third, overexpression of recombinant MUP1 in the liver markedly reduced hyperglycemia and glucose intolerance in three different types of diabetes. Fourth, overexpression of MUP1 suppressed the expression of both gluconeogenic and lipogenic genes in the liver. Fifth, recombinant MUP1 directly inhibited the hepatic gluconeogenic program in primary hepatocyte cultures.

It is less likely that MUP1 regulates metabolism by enhancing insulin sensitivity. Overexpression of MUP1 markedly reduced blood glucose levels and glucose intolerance in *db/db* mice but marginally enhanced insulin sensitivity. MUP1 also significantly reduced hyperglycemia and glucose intolerance in insulin-deficient type 1 diabetes. In addition, MUP1 overexpression neither significantly altered plasma insulin levels nor enhanced insulin signaling. In contrast, MUP1 attenuated the ability of cAMP analog (glucagon mimetic) and dexamethasone (glucocorticoid mimetic) to stimulate hepatic glucose production. Therefore, MUP1 may reduce blood glucose levels by inhibiting the hyperglycemic response to counter-regulatory hormones.

Recombinant MUP1 directly inhibited glucose production and the expression of gluconeogenic genes in primary hepatocyte cultures. These observations raise the possibility that MUP1 may suppress the hepatic glucose production by activating its own cognate receptor(s) expressed in hepatocytes. Consistent with this idea, urinary MUPs also directly stimulate sensory neurons in the vomeronasal organ (10). Additionally, circulatory MUP1 binds to lipophilic molecules, and these lipophilic molecules may directly regulate glucose and/or lipid metabolism. Therefore, MUP1 may regulate glucose and lipid metabolism indirectly by modulating the activity of these bioactive lipophilic molecules.

The liver is likely to be a physiological target of MUP1. MUP1 inhibited the expression of key gluconeogenic (*e.g.* G6Pase) and lipogenic genes (*e.g.* SCD1, FAS, ChREBP, and PPAR γ) in livers of *db/db* mice. Moreover, MUP1 also directly suppressed both basal and counter-regulatory hormone-stimulated glucose production in cultured hepatocytes. Therefore, MUP1 may regulate systemic glucose and lipid metabolism through paracrine/autocrine regulation of hepatocyte metabolic activity.

In contrast to MUP1, retinol-binding protein-4 (RBP4) and lipocalin-2, two other lipocalin family members, promote insulin resistance and hyperglycemia (21–23). Glucose metabolism is likely to be regulated by a balance between the MUP1 group and the RBP4/lipocalin-2 group of lipocalin family members. Interestingly, the human genome contains a gene (accession number: XM_001723632), which is predicted to encode a MUP1-like molecule (designated as hMUP). hMUP is ~50% identical to mouse MUP1 in amino acid sequence. We predict that hMUP also regulates glucose metabolism by a similar mechanism as MUP1.

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