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A Liver-Derived Secretory Protein, Selenoprotein P, C auses Insulin Resistance

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SUMMARY

The liver may regulate glucose homeostasis by modulating the sensitivity/resistance of peripheral tissues to insulin, by way of the production of secretory proteins, termed hepatokines. Here, we demonstrate that selenoprotein P (SeP), a liver-derived secretory protein, causes insulin resistance. Using serial analysis of gene expression (SAGE) and DNA chip methods, we found that hepatic SeP mRNA levels correlated with insulin resistance in humans. Administration of purified SeP impaired insulin signaling and dysregulated glucose metabolism in both hepatocytes and myocytes. Conversely, both genetic deletion and RNA interference-mediated knockdown of SeP improved systemic insulin sensitivity and glucose tolerance in mice. The metabolic actions of SeP were mediated, at least partly, by inactivation of adenosine monophosphate-activated protein kinase (AMPK). In summary, these results demonstrate a previously unrecognized role of SeP in the regulation of glucose metabolism and insulin sensitivity, and suggest that SeP may be a therapeutic target for type 2 diabetes.

INTRODUCTION

Insulin resistance is an underlying feature of people with type 2 diabetes and metabolic syndrome (Saltiel and Kahn, 2001), but is also associated with risk for cardiovascular diseases (Despres et al., 1996) and contributes to the clinical

manifestations of nonalcoholic steatohepatitis (Ota et al., 2007). In an insulin-resistant state, impaired insulin action promotes hepatic glucose production and reduces glucose uptake by peripheral tissues, resulting in hyperglycemia. The molecular mechanisms underlying insulin resistance are not fully understood, but are now known to be influenced by the secretion of tissue-derived factors, traditionally considered separate from the endocrine system. Recent work in obesity research, for example, has demonstrated that adipose tissues secrete a variety of proteins, known as adipocytokines (Friedman and Halaas, 1998; Maeda et al., 1996; Scherer et al., 1995; Steppan et al., 2001; Yang et al., 2005), which can either enhance or impair insulin sensitivity, thereby contributing to the development of insulin resistance.

SeP (in humans encoded by the *SEPP1* gene) is a secretory protein primarily produced by the liver (Burk and Hill, 2005; Carlson et al., 2004). It contains 10 selenocysteine residues, and functions as a selenium supply protein (Saito and Takahashi, 2002). However, the role of SeP in the regulation of glucose metabolism and insulin sensitivity has not yet been established. Furthermore, the clinical significance of SeP in human diseases has not been well defined, although studies of SeP knockout mice showed SeP deficiency to be associated with neurological injury and low fertility (Hill et al., 2003; Schomburg et al., 2003).

The liver plays a central role in glucose homeostasis and is also the site for the production of various secretory proteins. For example, recent work in our laboratory has revealed that genes encoding secretory proteins are abundantly expressed in the livers of people with type 2 diabetes (Misu et al., 2007). Moreover, genes encoding angiogenic factors, fibrogenic factors, and redox-associated factors were differentially expressed in the livers of people with type 2 diabetes (Takamura et al., 2004; Takeshita et al., 2006), possibly contributing to the pathophysiology of type 2 diabetes and its clinical manifestations. Based on these findings, we hypothesize that, analogous to adipose tissues, the liver may also contribute to the development of type 2 diabetes and insulin resistance, through the production of secretory proteins, termed hepatokines.

RESULTS

Identification of a Hepatic Secretory Protein Involved in Insulin Resistance

To identify hepatic secretory proteins involved in insulin resistance, we performed liver biopsies in humans and conducted a comprehensive analysis of gene expression profiles, using two distinct methods. First, we obtained human liver samples from five patients with type 2 diabetes and five non-diabetic subjects who underwent surgical procedures for malignant tumors, and we subjected them to serial analysis of

gene expression (SAGE) (Velculescu et al., 1995). Consequently, we identified 117 genes encoding putative secretory proteins with expression levels in people with type 2 diabetes, 1.5-fold or greater higher than those in normal subjects. Next, we obtained ultrasonography-guided percutaneous needle liver biopsies from 10 people with type 2 diabetes and seven normal subjects (Table S1), and we subjected them to DNA chip analysis to identify genes whose hepatic expression was significantly correlated with insulin resistance (Table S2). We performed glucose clamp experiments on these human subjects and measured the metabolic clearance rate (MCR) of glucose (glucose infusion rate divided by the steady-state plasma glucose concentration) as a measure of systemic insulin sensitivity. As a result, we found that *SEPP1* expression levels were up-regulated eight-fold in people with type 2 diabetes compared with normal subjects, as determined by SAGE (Table S2). Additionally, there was a negative correlation between hepatic *SEPP1* mRNA levels and the MCR of glucose, indicating that elevated hepatic *SEPP1* mRNA levels were associated with insulin resistance (Figure 1A). As a corollary, we found a positive correlation between the levels of hepatic *SEPP1* mRNA and post-loaded or fasting plasma glucose (Figures 1B and 1C).

Elevation of SeP in Type 2 Diabetes

To characterize the role of SeP in the development of insulin resistance, we measured serum SeP levels in human samples (Table S3), using enzyme-linked immunosorbent assays (ELISA), as described previously (Saito et al., 2001). Consistent with elevated hepatic *SEPP1* mRNA levels, we found a significant positive correlation between serum SeP levels and both fasting plasma glucose and hemoglobin A_{1c} (HbA_{1c}) levels (Figures 1D and 1E). HbA_{1c} is a clinical marker of protein glycation due to hyperglycemia, and elevated HbA_{1c} levels generally reflect poor glucose control over a 2–3-month period. Additionally, serum levels of SeP were significantly elevated in people with type 2 diabetes compared with normal subjects (Figure 1F, Table S4). Similar to data derived from clinical specimens, in rodent models of type 2 diabetes, including OLETF rats and KKAy mice, hepatic *Sepp1* mRNA and serum SeP levels were elevated (Figures 1G-1J, and Table S5).

SeP Expression in Hepatocytes Is Regulated by Glucose, Palmitate, and Insulin

To clarify the pathophysiology contributing to the hepatic expression of SeP in type 2 diabetes, we investigated the effects of nutrient supply on *Sepp1* mRNA expression in cultured hepatocytes. We found that the addition of glucose or palmitate upregulated *Sepp1* expression, whereas insulin downregulated it in a dose- and time-dependent manner (Figures 2A, 2C, 2E, 2F). Similar effects on SeP protein levels were observed in

primary mouse hepatocytes (Figures 2B, 2D, 2G). Consistent with the negative regulation of *Sepp1* by insulin in hepatocytes, *Sepp1* mRNA levels were elevated in the livers of fasting C57BL6J mice, compared with those that had been fed (Figure 2H). Thus, multiple lines of evidence suggest that elevated SeP is associated with the development of insulin resistance.

SeP Impairs Insulin Signaling and Dysregulates Glucose Metabolism *in Vitro*

Because there is no existing cell culture or animal model in which SeP is over-expressed, we purified SeP from human plasma using chromatographic methods (Saito et al., 1999; Saito and Takahashi, 2002) to examine the effects of SeP on insulin-mediated signal transduction. Treatment of primary hepatocytes with purified SeP induced a reduction in insulin-stimulated phosphorylation of insulin receptor (IR), and Akt (Figures 3A and 3B). SeP exerts its actions through an increase in cellular glutathione peroxidase (Saito and Takahashi, 2002). Co-administration of BSO, a glutathione synthesis inhibitor, rescued cells from the inhibitory effects of SeP (Figure 3C). Moreover, SeP increased phosphorylation of IRS1 at Ser307, the downregulator of tyrosine phosphorylation of IRS (Fig. S1A). Similar effects of SeP were also observed in C2C12 myocytes (Fig. S1B). Next, we assessed whether SeP dysregulated cellular glucose metabolism. In H4IIHEC hepatocytes, treatment with SeP up-regulated mRNA

expression of *Pck1* and *G6pc*, key gluconeogenic enzymes, resulting in a 30% increase in glucose release in the presence of insulin (Figures 3D-3F). Treatment with SeP alone had no effects on the levels of mRNAs encoding gluconeogenic enzymes or on glucose production in the absence of insulin, suggesting that SeP modulates insulin signaling. Additionally, treatment with SeP induced a reduction in insulin-stimulated glucose uptake in C2C12 myocytes (Figure 3G). These *in vitro* experiments indicate that, at physiological concentrations, SeP impairs insulin signal transduction and dysregulated cellular glucose metabolism.

SeP Impairs Insulin Signaling and Disrupts Glucose Homeostasis *in Vivo*

To examine the physiological effects of SeP *in vivo*, we treated female C57BL/6J mice with two intraperitoneal injections of purified human SeP (1 mg/kg body weight), 12 and 2 h before the experiments. Injection of purified human SeP protein resulted in serum levels of 0.5–1.5 μg/mL (data not shown). These levels correspond to the incremental change of SeP serum levels in people with normal glucose tolerance to those with type 2 diabetes (Saito et al., 2001). Glucose and insulin tolerance tests revealed that treatment of mice with purified SeP induced glucose intolerance and insulin resistance (Figures 3H and 3I). Blood insulin levels were significantly elevated in SeP-injected mice, although those of glucagon and GLP-1 were unaffected during a glucose tolerance

test (Fig. S1C). Western blot analysis showed a reduction in insulin-induced serine phosphorylation of Akt in both liver and skeletal muscle of SeP-injected mice (Figures 3J and 3K). Hyperinsulinemic-euglycemic clamp studies showed that treatment with SeP significantly increased endogenous glucose production and decreased peripheral glucose disposal (Figures S1D, 3L-M). Additionally, serum levels of injected human SeP protein negatively correlated with rates of peripheral glucose disposal (Fig. S1E). These data indicate that SeP impairs insulin signaling in the liver and skeletal muscle and induces glucose intolerance *in vivo*.

Knockdown of *Sepp1* in Liver Improves Glucose Intorelance and Insulin Resistance in Mice with Type 2 Diabetes

To determine whether knockdown of endogenous *Sepp1* enhances insulin signaling, we transfected H4IIEC hepatocytes with *Sepp1*-specific siRNA and observed a reduction in endogenous *Sepp1* mRNA and SeP protein levels (Figures 4A and 4B). Insulin-stimulated serine phosphorylation of Akt was enhanced in these treated cells (Figure 4C). Similarly, delivery of *Sepp1*-specific siRNAs into KKAy mice using a hydrodynamic transfection method (McCaffrey et al., 2002; Zender et al., 2003) resulted in a 30% reduction in SeP protein levels in the liver and blood (Figures 4D–G, S2).

Knockdown of *Sepp1* improved both glucose intolerance (Figures 4H and 4I) and insulin resistance (Figures 4J and 4K) in KKAy mice.

SeP-Deficient Mice Show Improved Glucose Tolerance and Enhanced Insulin Signaling in Liver and Muscle

We further confirmed the long-term effects of lowered SeP using *Sepp1* knockout mice (Hill et al., 2003). SeP knockout mice were viable and displayed normal body weights when maintained on a selenium-sufficient diet. Body weight, food intake, and O_2 consumption were unaffected by SeP knockout (Figures S3A and S3B). Lipid accumulation in the liver and adipose tissues was also unaffected (Figure 5A). However, postprandial plasma levels of insulin were reduced in *Sepp1*$^{-/-}$ mice, although blood glucose levels remained unchanged (Figures 5B and 5C). Glucose loading test revealed that *Sepp1*$^{-/-}$ mice showed improved glucose tolerance (Figure 5D). Insulin loading test revealed that *Sepp1*$^{-/-}$ mice showed lower blood glucose levels 60 min after insulin injection (Figure 5E). Insulin signaling, including phosphorylation of Akt and insulin receptor, was enhanced in the liver and skeletal muscle of *Sepp1*$^{-/-}$ mice (Figures 5F-5K). Additionally, *Sepp1*$^{+/-}$ tended to show enhanced insulin sensitivity. Plasma levels of glucagon, active GLP-1, and total GIP were unaffected by the loss of SeP in both fasted and fed mice (Figure S3C), suggesting that SeP dysregulated glucose metabolism *in vivo*

primarily by modulating the insulin pathway, but not by affecting other hormones, including glucagon, GLP-1, and GIP.

SeP Deficiency Attenuates Adipocyte Hypertrophy and Insulin Resistance in Dietary Obese Mice

To determine whether SeP deficiency reduces insulin resistance caused by diet-induced obesity, we fed SeP knockout mice a high-fat, high-sucrose diet (HFHSD) that is known to induce obesity, insulin resistance, and steatosis (Maeda et al., 2002). HFHSD tended to induce body weight gains in wild-type and *Sepp1*-deficient mice, although there was no significance between the three groups of animals (Figure 6A). Daily food intake was significantly increased in *Sepp1*$^{-/-}$ mice compared with wild-type animals (Figure 6B). Basal energy expenditure, as measured by O_2 consumption through indirect calorimetry, was also increased in *Sepp1*$^{-/-}$ mice (Figure 6C). Liver triglyceride content and epididymal fat mass were unaffected by *Sepp1* gene deletion (Figures S4A and 6D). However, diet-induced hypertrophy of adipocytes was attenuated in *Sepp1*$^{-/-}$ mice (Figures 6E, 6F, and S4B). Additionally, serum levels of free fatty acid and insulin were significantly reduced in these animals (Figures 6G-6I). Glucose and insulin loading tests revealed that *Sepp1*$^{-/-}$ mice were protected against glucose intolerance and insulin resistance even when on an obesity-inducing diet (Figures 6J and 6K).

SeP Reduces Phosphorylation of AMPKα both *in Vitro* and *in Vivo*

Adenosine monophosphate-activated protein kinase (AMPK) is a serine/threonine kinase that phosphorylates a variety of energy-associated enzymes and functions as a metabolic regulator that promotes insulin sensitivity (Kahn et al., 2005). In this study, we found that SeP treatment reduced phosphorylation of AMPKα and ACC in both H4IIEC hepatocytes and mouse liver (Figures S5A and 7A). Fatty acid β oxidation and β oxidation-related gene expression were also suppressed by SeP (Figures S5B-S5D). The levels of AMP and ATP were unchanged in hepatocytes treated with SeP (Figure S5E). In contrast, *Sepp1*-deficient mice exhibited increased phosphorylation of AMPKα and ACC in the liver (Figure 7B). To determine whether AMPK pathways were involved in the action of SeP, we infected H4IIEC hepatocytes with an adenovirus encoding dominant-negative (DN) or constitutively active (CA) AMPK. Transduction with DN-AMPK reduced insulin-stimulated Akt phosphorylation such that it could not be further decreased by SeP (Figures 7C–7E). In contrast, when CA-AMPK was over-expressed, SeP was unable to impair insulin-stimulated Akt phosphorylation (Figures 7F–H). Additionally, co-administration of 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAR), a known activator of AMPK, rescued cells from the inhibitory effects of SeP on insulin signaling (Figure 7I). These results suggest that reduced

phosphorylation of AMPK mediates, at least in part, the inhibitory effects of SeP on insulin signal transduction. Next, we examined the effects of SeP on some of the proteins that regulate the phosphorylation of AMPK. SeP dose-dependently increased the levels of protein phosphatase 2C (PP2C), a negative regulator of AMPK phosphorylation, in H4IIEC hepatocytes (Figure 7J). Expression of LKB1 and CaMKKβ, two positive regulators of AMPK, was unaffected by SeP treatment.

DISCUSSION

A Liver-Derived Secretory Protein, SeP, Causes Insulin Resistance

Our research reveals that hepatic overproduction of SeP contributes to the development of insulin resistance in the liver and skeletal muscle (Fig. S5F). The liver plays a central role in glucose homeostasis, mainly via glycogen storage and glucose release into the blood stream. In addition, the liver is a major site for the production of secretory proteins. Therefore, we hypothesized that the liver would maintain glucose homeostasis by producing liver-derived secretory protein(s) termed hepatokines. In fact, several studies have shown that hepatic secretory factors, including the angiopoietin-like protein family (Oike et al., 2005; Xu et al., 2005) and fetuin-A (Auberger et al., 1989; Srinivas et al., 1993), are involved in insulin sensitivity. However, we speculated that the

identification of the liver-derived proteins that directly contribute to the pathogenesis of insulin resistance or type 2 diabetes may not be adequate. Specifically, our comprehensive approach using global gene expression analyses revealed that numerous genes encoding secretory proteins are expressed and altered in the human type2 diabetic liver (Misu et al., 2007). Thus, by comparing the expression levels and clinical parameters for glycemic control and insulin resistance we selected candidate genes for liver-produced secretory proteins that cause insulin resistance. The current study sheds light on a previously underexplored function of the liver that is similar to adipose tissue; the liver may participate in the pathogenesis of insulin resistance through hormone secretion.

Suppression of SeP Expression by Insulin in Hepatocytes

Our results indicate that insulin negatively regulates SeP expression in hepatocytes. These findings are consistent with recent reports that the SeP promoter is a target of FoxO (forkhead box, class O) and PGC-1α (peroxisome proliferator-activated receptor-γ coactivator 1α), both of which are negatively regulated by insulin in hepatocytes (Speckmann et al., 2008; Walter et al., 2008). Consistent with these findings *in vitro*, we showed that hepatic SeP expression was up-regulated in mice in the fasting state. Under hypoinsulinemic conditions, such as a fasting state, up-regulation of SeP

might prevent hypoglycemia by decreasing glucose uptake in peripheral tissues and by increasing hepatic glucose production. Our results raise the possibility that the liver regulates systemic insulin sensitivity by sensing blood insulin levels and altering the production of SeP.

SeP Decreases Phosphorylation of AMPK and ACC in Hepatocytes

Identification of SeP receptor(s) in insulin-target organs is necessary to clarify the action mechanisms of SeP. Several lines of evidence have shown that apolipoprotein E receptor 2 (ApoER2) functions as an SeP receptor in the testis (Olson et al., 2007) and brain (Burk et al., 2007), both by acting as a cellular uptake receptor and by inducing intracellular signaling (Masiulis et al., 2009). It remains unknown whether ApoER2 acts as the SeP receptor in the liver or skeletal muscle. However, in this study, technical difficulties in the identification of a SeP receptor(s) led us to screen for well-established pathways associated with metabolic derangement to clarify the specific mechanisms of SeP action. As a result, our experiments reveal that SeP reduces phosphorylation of AMPK and its target ACC in H4IIEC hepatocytes and the livers of C57BL6J mice, possibly in an AMP/ATP ratio-independent manner. AMPK functions as a regulator of cellular energy homeostasis (Kahn et al., 2005) and mediates some effects of peripheral hormones such as leptin (Minokoshi et al., 2002) and adiponectin (Yamauchi et al.,

2002); however, the mechanisms by which these adipokines alter AMPK phosphorylation are not fully understood. Our present findings demonstrate that SeP increases the levels of PP2C in H4IIEC hepatocytes. PP2C is a phosphatase that inactivates AMPK by dephosphorylating a threonine residue (Thr172) that lies in its α-catalytic subunit (Davies et al., 1995). Tumor necrosis factor (TNF)-α, a representative inflammatory cytokine linked to insulin resistance, is known to reduce AMPK phosphorylation by up-regulating PP2C (Steinberg et al., 2006). Similar to TNF-α, SeP may reduce AMPK phosphorylation, at least partly, by up-regulating PP2C. Further characterization of SeP and SeP-receptor-mediated interactions will provide insights into the involvement of SeP in PP2C up-regulation and AMPK dephosphorylation.

Mechanism Underlying SeP-Mediated Insulin Resistance Varies between Liver and Skeletal Muscle

Given that plasma SeP is derived mainly from the liver (Carlson et al., 2004), our results suggest that AMPK mediates, at least in part, the autocrine/paracrine action of SeP. One limitation of our study is that the mechanism by which SeP acts on skeletal muscle remains unknown. Unlike in the liver, SeP-induced inhibitory effects on AMPK were not observed in either the skeletal muscle of C57BL6J mice or C2C12 myocytes (data not shown). Additionally, we showed that SeP reduces tyrosine phosphorylation of insulin

receptors in primary hepatocytes. In contrast, SeP acts on serine phosphorylation of IRS1, but not tyrosine phosphorylation of insulin receptors, in C2C12 myocytes (data not shown). These results suggest that SeP disrupts the insulin signal cascade at different levels between hepatocytes and myocytes. SeP might induce insulin resistance in skeletal muscle, possibly through AMPK-independent pathways. The mechanisms that connect SeP to insulin resistance likely exhibit tissue specificity.

We showed that SeP heterozygous mice have no phenotype in glucose- and insulin-loading tests, whereas a 30% decrease in SeP levels caused by the injection of siRNA improves glucose tolerance and insulin resistance in KKAy mice. In general, multiple compensatory changes are observed in knockout mice, because the target gene has been absent since conception. In contrast, compensation may be inadequate in adult animals in which the target gene has been knocked down using siRNA. In fact, real-time PCR analysis showed that expression of the gene encoding IL-6, a representative inflammatory cytokine linked to insulin resistance, shows compensatory upregulation in the liver of *Sepp1*$^{-/+}$ mice, but not in *Sepp1* siRNA-treated KKAy mice (Data not shown). Induction of IL-6 might compensate for the 50% reduction in SeP levels in *Sepp1*$^{-/+}$ mice.

Actions of SeP on the central nervous system may contribute to the *in vivo* phenotype. We did find that SeP-deficient mice fed a high-fat, high-sucrose diet display

increases in food intake and O2 consumption (Fig. 6B, 6C), suggesting that SeP acts on the central nervous system. Additionally, an earlier report described the co-localization of SeP and amyloid-β protein in the brains of people with Alzheimer's disease, suggesting the potential involvement of SeP in this condition's pathology (Bellinger et al., 2008). More recently, Takeda et al. reported that amyloid pathology in Alzheimer's disease may adversely affect diabetic phenotypes in mice (Takeda et al., 2010). Further experiments are necessary to determine whether the actions of SeP on the central nervous system involve the *in vivo* phenotype seen in this study.

We cannot exclude the possibility that the current phenotype in *Sepp1*-deficient mice is affected by the abnormal distribution of selenium. In fact, selenium levels in plasma and several tissues have been reported to be reduced in *Sepp1*-deficient mice fed a selenium-restricted diet (Schomburg et al., 2003). However, Burk et al. reported that the selenium levels in all tissues except the testis were unchanged in these mice fed a diet containing adequate amounts of selenium (Hill et al., 2003). In this study, we performed experiments using *Sepp1*-deficient mice fed a diet containing adequate amounts of selenium. Thus, we speculate that the effects of abnormal selenium distribution on our results in *Sepp1*-deficient mice may be insignificant.

A limitation of this study is that we could not match age, gender, or body weight completely between people with type 2 diabetes and normal subjects when comparing the serum SeP levels, due to the limited sample numbers. However, a previous large-scale clinical report showed that the age-, gender-, race-, and BMI-adjusted mean serum selenium levels were significantly elevated in participants with diabetes compared with those without diabetes in the US population (Bleys et al., 2007). Additionally, several lines of evidence showed that serum selenium levels are positively correlated with those of SeP in humans (Andoh et al., 2005; Persson-Moschos et al., 1998). In combination with our result, these reports lead us to speculate that serum SeP levels are also elevated in people with type 2 diabetes compared with normal subjects. However, additional large-scale clinical trials are needed to address this.

In summary, our experiments have identified SeP as a liver-derived secretory protein that induces insulin resistance and hyperglycemia. Our findings suggest that the secretory protein SeP may be a target for the development of therapies to treat insulin resistance-associated diseases, including type 2 diabetes.

EXPERIMENTAL PROCEDURES

Animals

Eight-week-old c57BL/6J mice were obtained from Sankyo Lab Service (Tokyo, Japan). Male Otsuka Long–Evans Tokushima Fatty (OLETF) rats and Long–Evans Tokushima Otsuka (LETO) rats were obtained from the Otsuka Pharmaceutical Tokushima Research Institute (Tokushima, Japan). OLETF rats have been established as an animal model of obesity-related type 2 diabetes (Kawano et al., 1992). Female KKAy mice were obtained from CLEA Japan (Tokyo, Japan). All animals were housed in a 12-h light/dark cycle and allowed free access to food and water. High fat and high sucrose diet (D03062301) was purchased from Research Diets (New Brunswick, NJ). The experiments with OLETF and LETO rats were performed using frozen blood and liver samples obtained in our previous study (Ota et al., 2007).

Purification of SeP

SeP was purified from human plasma using conventional chromatographic methods, as previously described (Saito et al., 1999; Saito and Takahashi, 2002). Homogeneity of purified human SeP was confirmed by analysis of both amino acid composition and sequence (Saito et al., 1999). Concentrations of purified SeP were measured by the

Bradford method, using bovine immunoglobulin G as a standard.

siRNA Injection into KKAy mice

Delivery of siRNA targeted to the liver was performed by tail vein injections into mice, using hydrodynamic techniques, as previously described (McCaffrey et al., 2002; Zender et al., 2003). For these experiments, KKAy mice at 7–8 weeks of age (31–33 g body weight) were used. Mice were anesthetized with pentobarbital, and 2 nmol of siRNA, diluted in 3 mL of PBS, were injected into the tail vein over 15–20 s. All siRNAs were purchased from Applied Biosystems (Silencer^{R} In Vivo Ready Pre-designed siRNA). *Sepp1* siRNAs with the following sequences were synthesized: mouse *Sepp1*: 5'-GGUGUCAGAACACAUCGCAtt-3' (sense). Negative control siRNA was also used and had no significant homology with any known gene sequences in mouse, rat, or human. Glucose and insulin loading tests were performed 2–7 days following injection of mice with siRNA.

SeP knockout mice

SeP knockout mice were produced by homologous recombination using genomic DNA cloned from an Sv-129 P1 library, as described previously(Hill et al., 2003). As female

SeP knockout mice had inconsistent phenotypes, only male mice were used in this study.

Statistical analyses

All data were analyzed using the Japanese Windows Edition of the Statistical Package for Social Science (SPSS) Version 11.0. Numeric values are reported as the mean±SEM. Differences between two groups were assessed using unpaired two-tailed *t*-tests. Data involving more than two groups were assessed by analysis of variance (ANOVA). Glucose and insulin tolerance tests were examined using repeated measures ANOVA.

Accession codes

Microarray data have been deposited in Gene Expression Omnibus: GSE23343.

SUPPLEMENTAL INFORMATION

Supplemental Data include Supplemental Experimental Procedures, Supplemental references, five figures, and five tables.

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Figure legends

Figure 1. **Elevation of serum SeP levels and hepatic *Sepp1* expression in type 2 diabetes**

(A-C) Individual correlations between hepatic *SEPP1* mRNA levels and metabolic clearance rate (MCR) of glucose (A), post-loaded plasma glucose levels (B), and fasting plasma glucose levels (C) in humans (n = 12-17). MCR = glucose infusion rate divided by the steady-state plasma glucose concentration, and is a measure of systemic insulin sensitivity. MCR values were determined by glucose clamp. *SEPP1* mRNA levels were quantified using DNA chips.

(D and E**)** Correlations between serum levels of SeP and fasting plasma glucose levels (D) and HbA_{1c} (E) in people with type 2 diabetes (n = 35).

(F**)** Serum levels of SeP in people with type 2 diabetes and healthy subjects (n = 9-12). Age and body weight were not significantly different b etween the two groups. Data represents the means ± SEM from two groups. *$p < 0.05$.

(G and H) Hepatic *Sepp1* mRNA levels in an animal model of type 2 diabetes (n = 5-6).

(I and J) Serum SeP levels in an animal model of type 2 diabetes. SeP was detected by Western blotting. Coomassie Brilliant Blue (CBB)-stained gel is used as a control for

protein loading. Graphs display the results of densitometric quantification, normalized to CBB-stained proteins ($n = 5$). Data represent the mean ± SEM from five to six mice per group. $*p < 0.05$, $**p < 0.01$.

Figure 2. **SeP expression is regulated by glucose, palmitate, and insulin**

(A) *Sepp1* mRNA levels in H4IIEC hepatocytes treated with glucose or mannitol (30 mM) for 6 h ($n = 4$).

(B) SeP protein levels in primary hepatocytes treated with glucose or mannitol for 6 h.

(C) *Sepp1* mRNA levels in H4IIEC hepatocytes treated with palmitate (0.25 mM) for 16 h ($n = 5$).

(D) SeP protein levels in primary hepatocytes treated with palmitate (0.25 mM) for 16 h.

(E) *Sepp1* and *Pck1* mRNA levels in H4IIEC hepatocytes treated with various concentrations of insulin for 6 h ($n = 4$).

(F) *Sepp1* mRNA levels in H4IIEC hepatocytes treated with insulin (0.1 μg/mL) for the indicated periods of time ($n = 4$).

(G) SeP protein levels in primary hepatocytes treated with various concentrations of insulin for 6 h.

(H) Liver *Sepp1*, *Pck1*, and *G6pc* mRNA levels in C57BL/6J mice following fasting for

12 h and subsequent re-feeding ($n = 4$). Data in (A), (C), (E), and (F) represent the mean s ± SEM from four to five cells per group, and data in (H) represent the means ± SEM fr om four mice per group. $*p < 0.05$, $**p < 0.01$.

Figure 3. **SeP impairs insulin signaling *in vitro* and *in vivo***

(A and B) Effects of SeP on serine phosphorylation of Akt (A) and tyrosine phosphorylation of insulin receptor (B) in insulin-stimulated primary hepatocytes. Data represent the means ± SEM of three independent experiments. $*p < 0.05$, $**p < 0.01$ (vs. vehicle-treated cells). Primary hepatocytes were treated with SeP or vehicle for 24 h. Then, the cells were stimulated with 1 ng/mL insulin for 15 min.

(C) Effects of BSO on SeP-induced changes in insulin-stimulated Akt phosphorylation in primary hepatocytes.

(D and E) Effects of SeP on the expression of mRNAs encoding gluconeogenic enzymes in H4IIEC hepatocytes ($n = 5$).

(F) Release of glucose from H4IIEC hepatocytes treated with SeP for 24 h ($n = 6$).

(G) Effects of SeP on glucose uptake in C2C12 myocytes ($n = 6$).

(H and I) Glucose (H) and insulin (I) tolerance tests in mice injected with SeP or vehicle ($n = 5$). Glucose (1.5 g/kg body weight) or insulin (0.5 unit/kg body weight) was

administered intraperitoneally.

(J and K) Effects of SeP on serine phosphorylation of Akt in liver (J) and skeletal muscle (K) in mice injected with purified human SeP or vehicle. Mice (n = 3 or 4) were stimulated with insulin (administered intraperitoneally). At 20 min after insulin stimulation, mice were anesthetized and liver and hindlimb muscle samples removed for analysis.

(L) Time course of glucose infusion rate (GIR) during hyperinsulinemic–euglycemic clamp in mice injected with SeP or vehicle (n = 6).

(M) GIR, endogenous glucose production (EGP), and rate of glucose disposal (Rd) during hyperinsulinemic–euglycemic clamp (n = 6). C57BL/6J mice were twice injected intraperitoneally with purified human SeP (1 mg/kg body weight) or vehicle in H-M. Injections were administered 12 and 2 h before the each experiment. Data in (D), (E), (F) , and (G) represent the means ± SEM from five to six cells per group, and data in (H), (I) , (J), (K), (L), and (M) represent the means ± SEM from three to six mice per group. *p < 0.05, **p < 0.01 versus cells treated with vehicle in D-G. *p < 0.05, **p < 0.01 versus mice treated with vehicle in H–M.

Figure 4. ***Sepp1*** **knockdown in the liver improves insulin sensitivity**

(A) *Sepp1* mRNA levels in H4IIEC hepatocytes transfected with control or *Sepp1*-specific siRNA ($n = 4$).

(B) SeP protein production in H4IIEC hepatocytes transfected with *Sepp1*-specific siRNA. SeP production was detected in whole cell lysates by Western blotting.

(C) Effects of SeP knockdown on insulin-stimulated serine phosphorylation of Akt in H4IIEC hepatocytes. Data represent the mean±SEM of three independent experiments.

(D and E) Liver SeP production in KKAy mice injected with control or *Sepp1*-specific siRNA ($n = 6$). SeP protein levels were measured by Western blotting 4 days after injection of siRNA.

(F and G) Blood SeP levels in KKAy mice injected with siRNA. Blood samples were obtained 4 days after siRNA injection ($n = 6$).

(H–K) Intraperitoneal glucose (H and I) and insulin (J and K) tolerance tests in KKAy mice (n = 6-8) injected with control or *Sepp1*-specific siRNA. Glucose and insulin was administered at doses of 0.3 g/kg body weight and 4 units/kg body weight, respectively. Area under the curve (AUC) for blood glucose levels is shown in (I) and (K). Data in (A) represent the means ± SEM from four cells per group, and data in (E), (G), (H), (I), (J), and (K) represent the means ± SEM from six to eight mice per group. $*p < 0.05$ versus

cells transfected with control siRNA in (A) and (C). *$p < 0.05$, **$p < 0.01$ versus mice i njected with control siRNA in (E), (G), (H), (I), (J), and (K).

Figure 5. ***Sepp1*-deficient mice show improved glucose tolerance and enhanced insulin sensitivity**

(A) Hematoxylin-and-eosin-stained liver and epididymal fat sections from male *Sepp1*$^{+/-}$ and *Sepp1*$^{-/-}$ mice.

(B) Blood glucose levels in *Sepp1*-deficient mice ($n = 7$). The mice were fasted for 6 h.

(C) Blood insulin levels in *Sepp1*-deficient mice ($n = 7$).

(D and E) Intraperitoneal glucose (D) and insulin (E) tolerance tests in male *Sepp1*-deficient mice ($n = 7$). Glucose and insulin were administered at doses of 1.5 g/kg body weight and 4 units/kg body weight, respectively.

(F–K) Western blot analysis of phosphorylated Akt (pAkt) and phosphorylated insulin receptor (pIR) in liver (F–H) and skeletal muscle (I-K). Mice ($n = 6$) were stimulated with insulin (administered intraperitoneally). At 20 min after insulin stimulation, mice were anesthetized and liver and hindlimb muscle samples removed for analysis. Data in (B), (C), (D), (E), (G), (H), (J), and (K) represent the means ± SEM from six to seven mi ce per group. *$p < 0.05$, **$p < 0.01$ versus wild-type mice.

Figure 6. ***Sepp1*-deficient mice are protected from diet-induced insulin resistance and adipocyte hypertrophy**

(A) Body weight of *Sepp1*-deficient and wild-type mice fed a high-fat, high-sucrose diet (HFHSD; n = 4-8). Sixteen-week-old male mice fed a HFHSD for 16 weeks.

(B) Daily calorie intake in *Sepp1*-deficient and wild-type mice (n = 4–8).

(C) Energy expenditure (as measured by VO_2 consumption through indirect calorimetry; n = 4).

(D) Epididymal fat mass in *Sepp1*-deficient and wild-type mice fed HFHSD (n = 4–7).

(E) Hematoxylin-and-eosin-stained epididymal fat sections from wild-type and $Sepp1^{-/-}$ mice.

(F) Histogram showing adipocyte diameters. We determined adipocyte diameters by measuring at least 300 adipocytes randomly selected from four independent sections.

(G) Blood non-estimated fatty acid levels in *Sepp1*-deficient and wild-type mice fed HFHSD (n = 4–7).

(H) Blood glucose levels in *Sepp1*-deficient and wild-type mice fed HFHSD (n = 4–8).

(I) Blood insulin levels in *Sepp1*-deficient and wild-type mice fed HFHSD (n = 4–8).

Blood samples were obtained from mice fed a HFHSD for 16 weeks after a 12 hr fast in

(G-I).

(J) Intraperitoneal glucose tolerance tests in wild-type and *Sepp1*-deficient mice (n = 4-8). Glucose was administered at a dose of 0.3 g/kg body weight.

(K) Intraperitoneal insulin tolerance tests in wild-type and *Sepp1*-deficient mice (n = 5-10). Insulin was administered at a dose of 2.0 units/kg body weight). Data in (A), (B), (C), (D), (G), (H), (I), (J), and (K) represent the means ± SEM from four to ten mice per group. $*p < 0.05$, $**p < 0.01$ versus wild-type mice.

Figure 7. **SeP reduces phosphorylation of AMPK and ACC in hepatocytes**

(A) Phosphorylation of AMPK and ACC in the liver of mice injected with SeP or vehicl e. C57BL/6J mice were injected intravenously with purified human SeP (1 mg/kg body weight) or vehicle (phosphate-buffered saline). At 6 h after injection, the liver was removed.

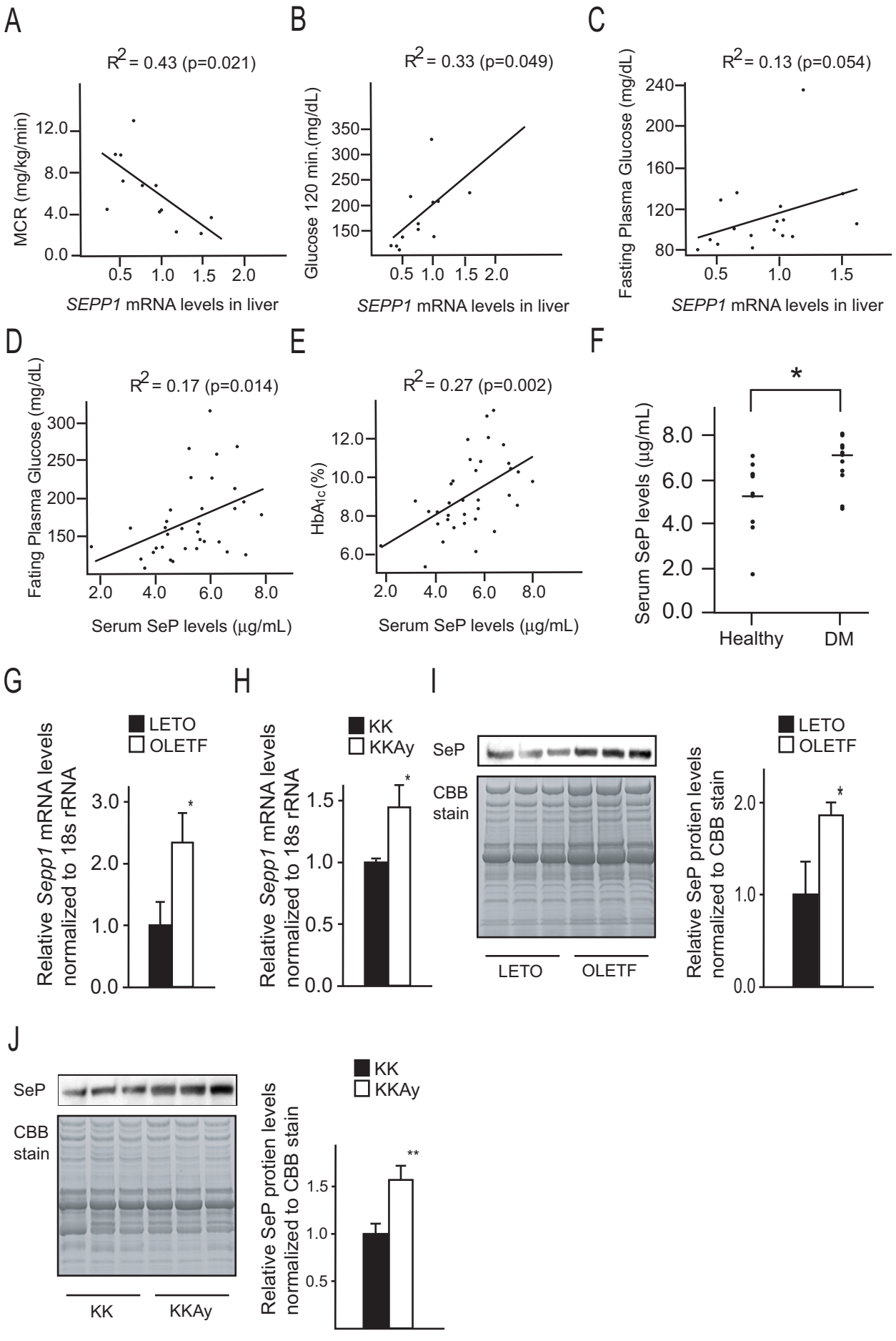
(B) Phosphorylation of AMPK and ACC in the liver of *Sepp1*-deficient mice after a 12 h r fast.

(C–E) Effects of dominant-negative AMPK on ACC phosphorylation (C) and

insulin-stimulated Akt phosphorylation (D and E) in H4IIEC hepatocytes treated with SeP. (F–H) Effects of constitutively active AMPK on ACC phosphorylation (F) and insulin-stimulated Akt phosphorylation (G and H) in H4IIEC hepatocytes treated with SeP.

(I) Effect of AICAR on SeP-induced insulin resistance in H4IIEC hepatocytes.

(J) Levels of PP2C, CaMKKβ, and LKB1 in H4IIEC hepatocytes treated with various concentrations of SeP for 12 h. Data in (E) and (H) represent the means ± SEM from thr ee independent experiments. $^{**}p < 0.01$ versus vehicle-treated cells.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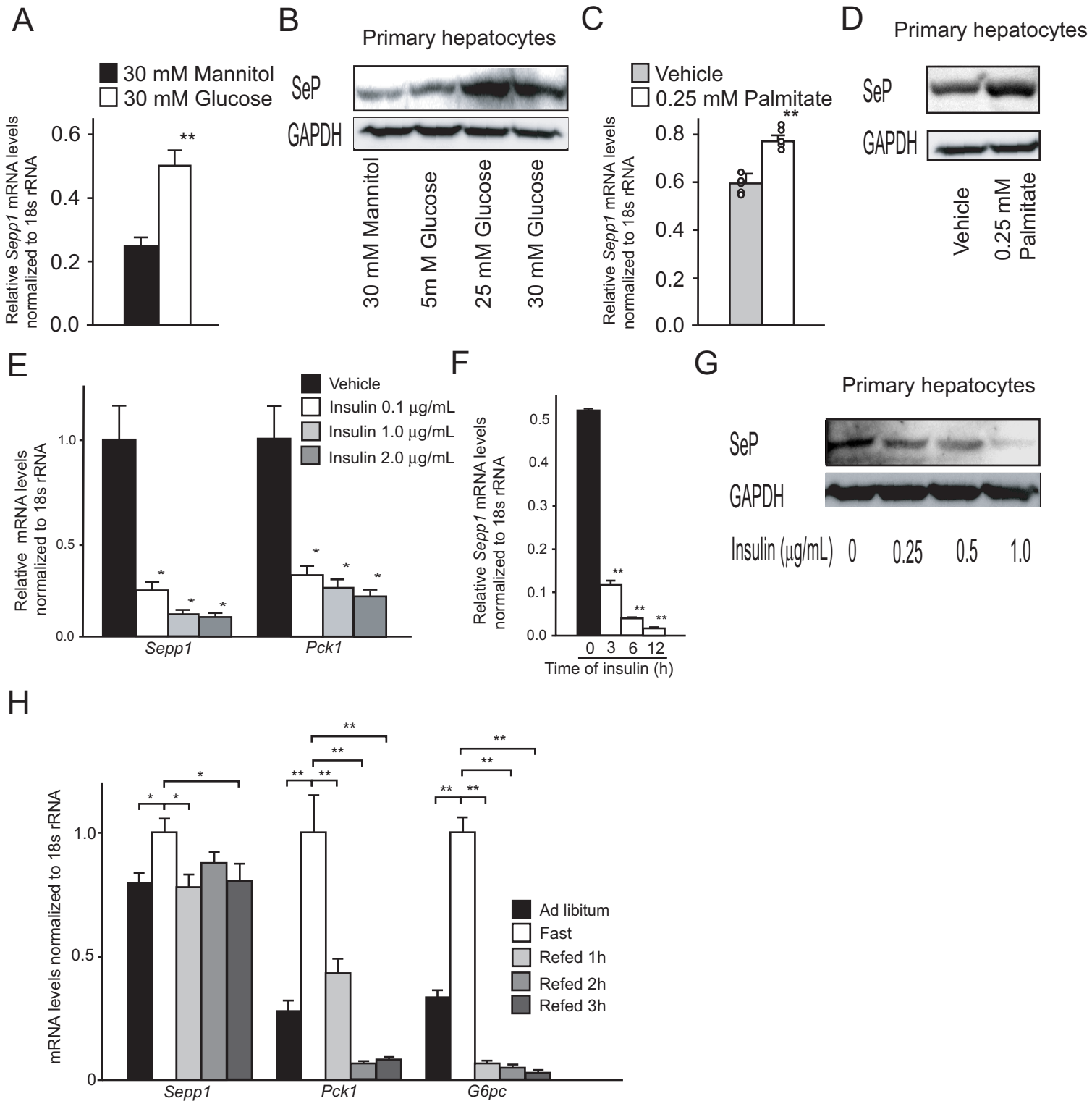
MCR (mg/kg/min)
12.0
8.0
4.0
0.0
0.5 1.0 1.5 2.0
SEPP1 mRNA levels in liver
B
R^2 = 0.33 (p=0.049)
Glucose 120 min.(mg/dL)
350
300
250
200
150
0.5 1.0 1.5 2.0
SEPP1 mRNA levels in liver
C
R^2 = 0.13 (p=0.054)
Fasting Plasma Glucose (mg/dL)
240
200
160
120
80
0.5 1.0 1.5
SEPP1 mRNA levels in liver
D
R^2 = 0.17 (p=0.014)
Fating Plasma Glucose (mg/dL)
300
250
200
150
2.0 4.0 6.0 8.0
Serum SeP levels (μg/mL)
E
R^2 = 0.27 (p=0.002)
HbA_{1c}(%)
12.0
10.0
8.0
6.0
2.0 4.0 6.0 8.0
Serum SeP levels (μg/mL)
F
*
Serum SeP levels (μg/mL)
8.0
6.0
4.0
2.0
0.0
Healthy
DM
G
LETO
OLETF
Relative *Sepp1* mRNA levels normalized to 18s rRNA
3.0
2.0
1.0
0.0
*
H
KK
KKAy
Relative *Sepp1* mRNA levels normalized to 18s rRNA
1.5
1.0
0.5
0.0
*
I
SeP
CBB stain
LETO
OLETF
LETO
OLETF
Relative SeP protien levels normalized to CBB stain
2.0
1.0
0.0
*
J
SeP
CBB stain
KK
KKAy
KK
KKAy
Relative SeP protien levels normalized to CBB stain
1.5
1.0
0.5
**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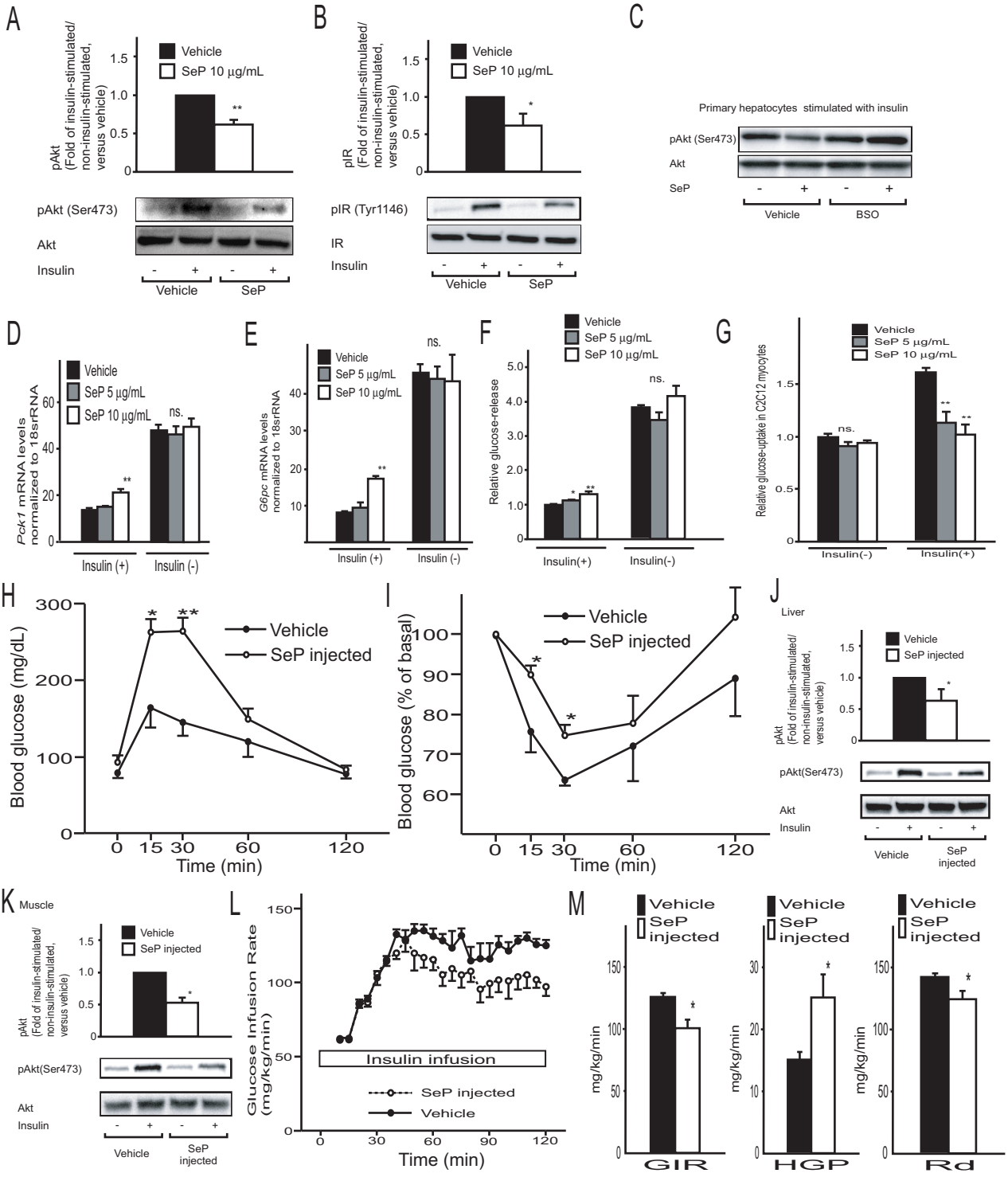
30 mM Mannitol
30 mM Glucose
Relative Sepp1 mRNA levels normalized to 18s rRNA
0.0
0.2
0.4
0.6
**
B
Primary hepatocytes
SeP
GAPDH
30 mM Mannitol
5m M Glucose
25 mM Glucose
30 mM Glucose
C
Vehicle
0.25 mM Palmitate
Relative Sepp1 mRNA levels normalized to 18s rRNA
0.0
0.2
0.4
0.6
0.8
**
D
Primary hepatocytes
SeP
GAPDH
Vehicle
0.25 mM Palmitate
E
Vehicle
Insulin 0.1 µg/mL
Insulin 1.0 µg/mL
Insulin 2.0 µg/mL
Relative mRNA levels normalized to 18s rRNA
0.0
0.5
1.0
Sepp1
Pck1
F
Relative Sepp1 mRNA levels normalized to 18s rRNA
0.0
0.1
0.2
0.3
0.4
0.5
0 3 6 12
Time of insulin (h)
G
Primary hepatocytes
SeP
GAPDH
Insulin (µg/mL) 0 0.25 0.5 1.0
H
mRNA levels normalized to 18s rRNA
0
0.5
1.0
Sepp1
Pck1
G6pc
Ad libitum
Fast
Refed 1h
Refed 2h
Refed 3h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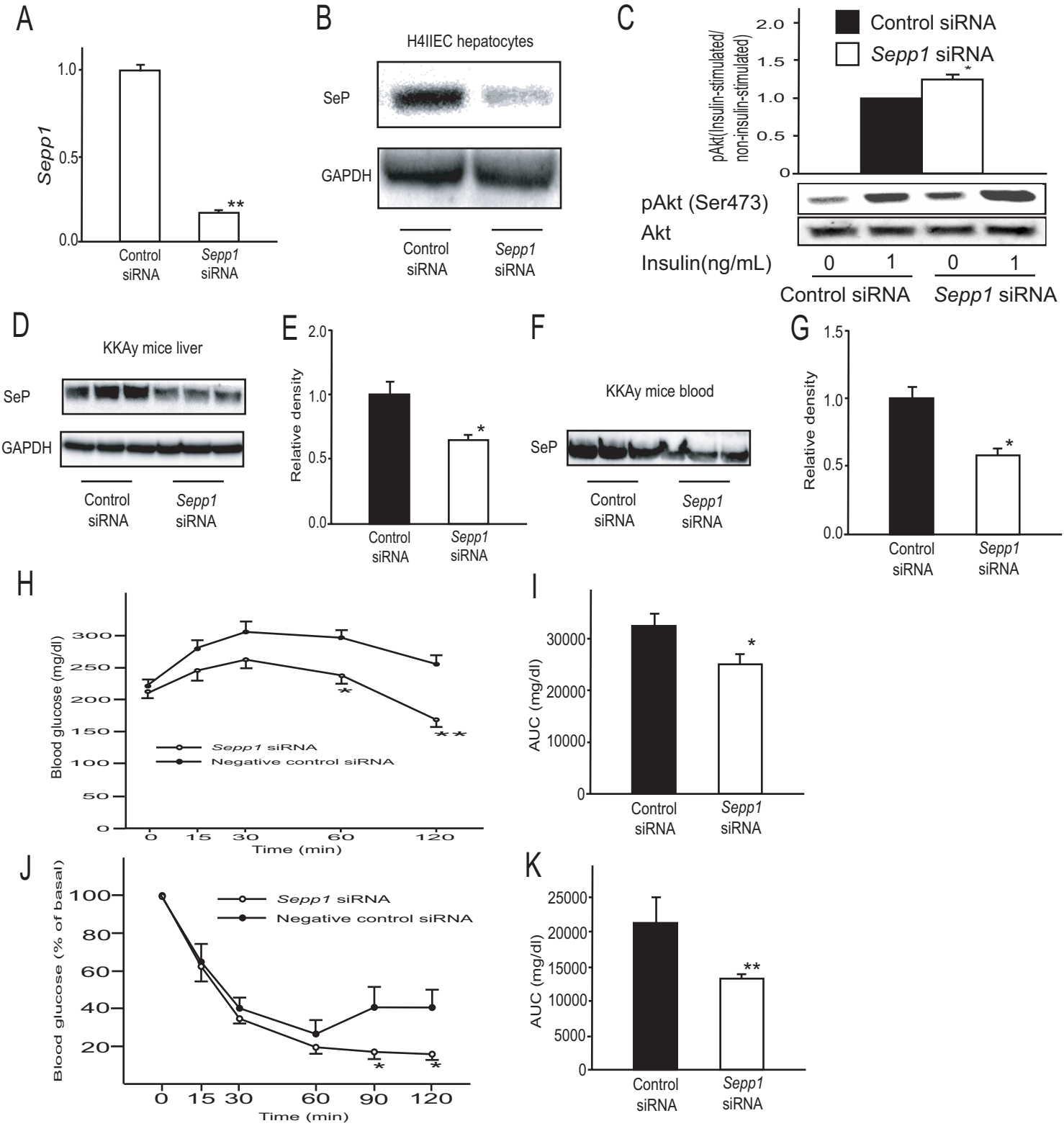
Vehicle
SeP 10 μg/mL
pAkt (Ser473)
Akt
Insulin
Vehicle
SeP
B
pIR (Fold of insulin-stimulated/ non-insulin-stimulated, versus vehicle)
pIR (Tyr1146)
IR
C
Primary hepatocytes stimulated with insulin
pAkt (Ser473)
Akt
SeP
Vehicle
BSO
D
Pck1 mRNA levels normalized to 18srRNA
SeP 5 μg/mL
Insulin (+)
Insulin (-)
ns.
E
G6pc mRNA levels normalized to 18srRNA
F
Relative glucose-release
Insulin(+)
Insulin(-)
G
Relative glucose-uptake in C2C12 myocytes
H
Blood glucose (mg/dL)
SeP injected
Time (min)
I
Blood glucose (% of basal)
J
Liver
K
Muscle
L
Glucose Infusion Rate (mg/kg/min)
Insulin infusion
M
GIR
HGP
Rd
mg/kg/min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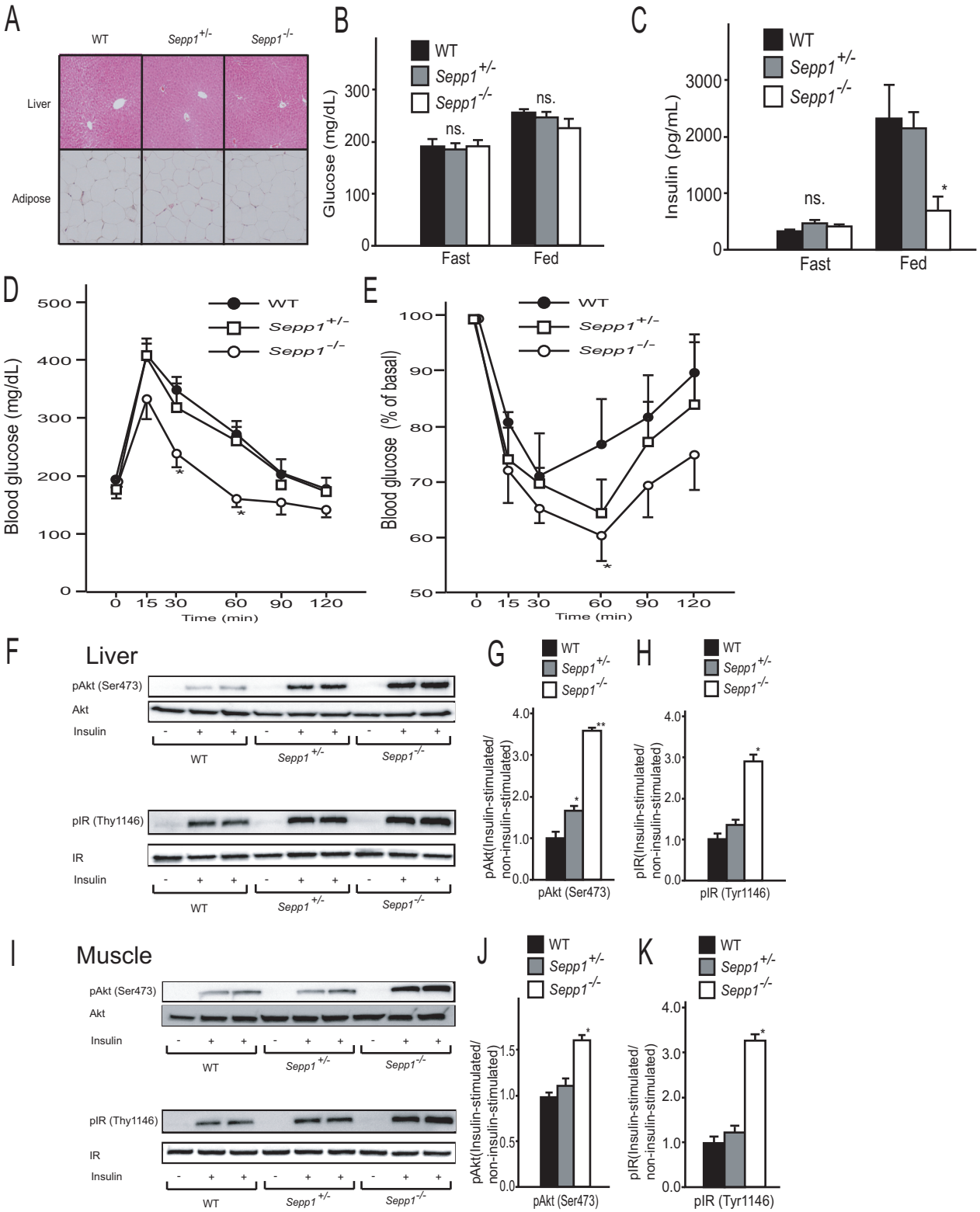
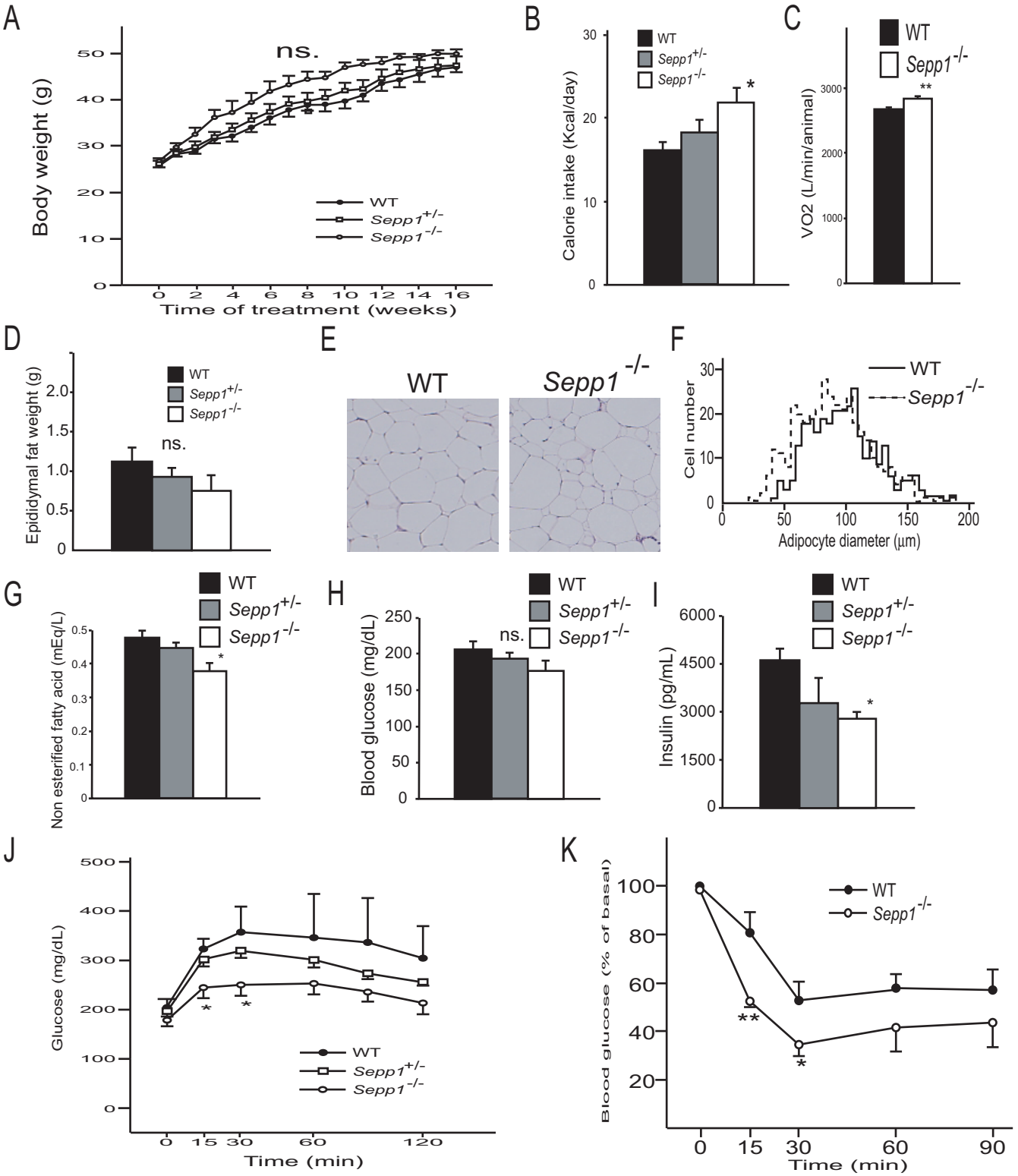
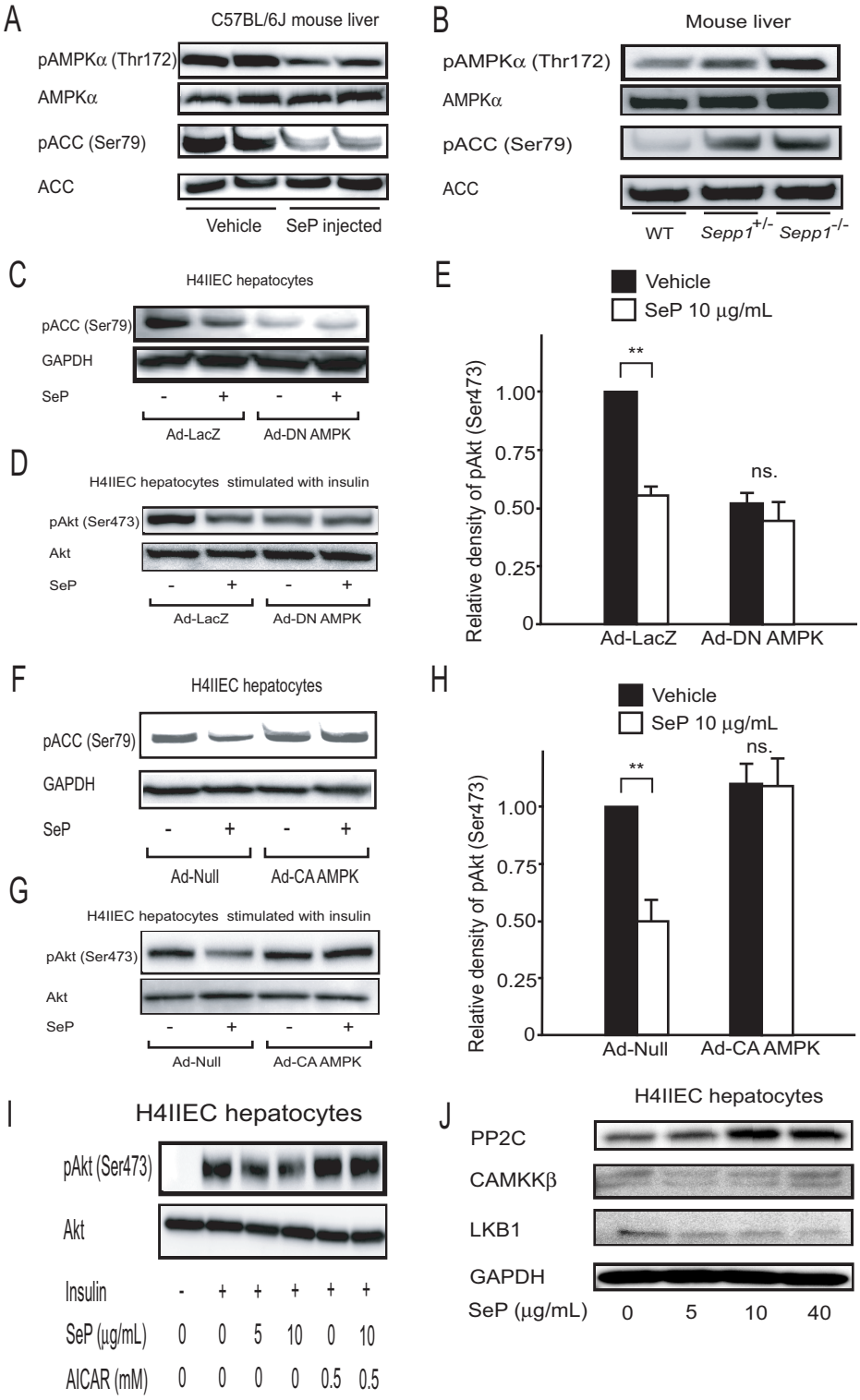
Sepp1
1.0
0.5
0.0
**
Control siRNA
Sepp1 siRNA
B
H4IIEC hepatocytes
SeP
GAPDH
Control siRNA
Sepp1 siRNA
C
pAkt(Insulin-stimulated/ non-insulin-stimulated)
2.0
1.5
1.0
0.5
0
Control siRNA
Sepp1 siRNA
*
pAkt (Ser473)
Akt
Insulin(ng/mL)
0 1 0 1
Control siRNA
Sepp1 siRNA
D
KKAy mice liver
SeP
GAPDH
Control siRNA
Sepp1 siRNA
E
Relative density
2.0
1.0
0.5
0.0
*
Control siRNA
Sepp1 siRNA
F
KKAy mice blood
SeP
Control siRNA
Sepp1 siRNA
G
Relative density
1.5
1.0
0.5
0.0
*
Control siRNA
Sepp1 siRNA
H
Blood glucose (mg/dl)
300
250
200
150
100
50
0
Sepp1 siRNA
Negative control siRNA
* **
0 15 30 60 120
Time (min)
I
AUC (mg/dl)
30000
20000
10000
0
*
Control siRNA
Sepp1 siRNA
J
Blood glucose (% of basal)
100
80
60
40
20
Sepp1 siRNA
Negative control siRNA
* *
0 15 30 60 90 120
Time (min)
K
AUC (mg/dl)
25000
20000
15000
10000
5000
0
**
Control siRNA
Sepp1 siRNA


A
WT
Sepp1+/-
Sepp1-/-
Liver
Adipose
B
WT
Sepp1+/-
Sepp1-/-
Glucose (mg/dL)
ns.
ns.
Fast
Fed
C
WT
Sepp1+/-
Sepp1-/-
Insulin (pg/mL)
ns.
*
Fast
Fed
D
WT
Sepp1+/-
Sepp1-/-
Blood glucose (mg/dL)
Time (min)
E
WT
Sepp1+/-
Sepp1-/-
Blood glucose (% of basal)
Time (min)
F
Liver
pAkt (Ser473)
Akt
Insulin
WT
Sepp1+/-
Sepp1-/-
pIR (Thy1146)
IR
Insulin
G
WT
Sepp1+/-
Sepp1-/-
pAkt(Insulin-stimulated/non-insulin-stimulated)
pAkt (Ser473)
H
WT
Sepp1+/-
Sepp1-/-
pIR(Insulin-stimulated/non-insulin-stimulated)
pIR (Tyr1146)
I
Muscle
pAkt (Ser473)
Akt
Insulin
WT
Sepp1+/-
Sepp1-/-
pIR (Thy1146)
IR
Insulin
J
WT
Sepp1+/-
Sepp1-/-
pAkt(Insulin-stimulated/non-insulin-stimulated)
pAkt (Ser473)
K
WT
Sepp1+/-
Sepp1-/-
pIR(Insulin-stimulated/non-insulin-stimulated)
pIR (Tyr1146)


A
Body weight (g)
ns.
WT
Sepp1+/-
Sepp1-/-
Time of treatment (weeks)
B
Calorie intake (Kcal/day)
C
VO2 (L/min/animal)
D
Epididymal fat weight (g)
E
WT
Sepp1-/-
F
Cell number
Adipocyte diameter (µm)
G
Non esterified fatty acid (mEq/L)
H
Blood glucose (mg/dL)
I
Insulin (pg/mL)
J
Glucose (mg/dL)
Time (min)
K
Blood glucose (% of basal)
Time (min)


A
C57BL/6J mouse liver
pAMPKα (Thr172)
AMPKα
pACC (Ser79)
ACC
Vehicle
SeP injected
B
Mouse liver
pAMPKα (Thr172)
AMPKα
pACC (Ser79)
ACC
WT
Sepp1+/-
Sepp1-/-
C
H4IIEC hepatocytes
pACC (Ser79)
GAPDH
SeP
- + - +
Ad-LacZ
Ad-DN AMPK
D
H4IIEC hepatocytes stimulated with insulin
pAkt (Ser473)
Akt
SeP
- + - +
Ad-LacZ
Ad-DN AMPK
E
Vehicle
SeP 10 μg/mL
Relative density of pAkt (Ser473)
1.00
0.75
0.50
0.25
0
**
ns.
Ad-LacZ
Ad-DN AMPK
F
H4IIEC hepatocytes
pACC (Ser79)
GAPDH
SeP
- + - +
Ad-Null
Ad-CA AMPK
G
H4IIEC hepatocytes stimulated with insulin
pAkt (Ser473)
Akt
SeP
- + - +
Ad-Null
Ad-CA AMPK
H
Vehicle
SeP 10 μg/mL
Relative density of pAkt (Ser473)
1.00
0.75
0.50
0.25
0
**
ns.
Ad-Null
Ad-CA AMPK
I
H4IIEC hepatocytes
pAkt (Ser473)
Akt
Insulin - + + + + +
SeP (μg/mL) 0 0 5 10 0 10
AICAR (mM) 0 0 0 0 0.5 0.5
J
H4IIEC hepatocytes
PP2C
CAMKKβ
LKB1
GAPDH
SeP (μg/mL) 0 5 10 40