Deletion of Cd39/Entpd1 results in hepatic insulin resistance.

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ABSTRACT

Objective: Extracellular nucleotides are important mediators of inflammatory responses and could also impact metabolic homeostasis. Type-2 purinergic (P2) receptors bind extracellular nucleotides and are expressed by major peripheral tissues responsible for glucose homeostasis. CD39/ENTPD1 is the dominant vascular and immune cell ecto-enzyme that hydrolyzes extracellular nucleotides to regulate purinergic signaling.

Research Design and Methods: We have studied Cd39/Entpd1 null mice to determine whether any associated changes in extracellular nucleotide concentrations influence glucose homeostasis.

Results: Cd39/Entpd1 null mice have impaired glucose tolerance and decreased insulin sensitivity with significantly higher plasma insulin levels. Hyperinsulinemic euglycemic clamp studies indicate altered hepatic glucose metabolism. These effects are mimicked in vivo by injection into wild-type mice of either exogenous ATP or an ecto-ATPase inhibitor, ARL-67156 and by exposure of hepatocytes to extracellular nucleotides in vitro. Increased serum Interleukin-1β, Interleukin-6, IFN-γ and TNF-α levels are observed in Cd39/Entpd1 null mice in keeping with a pro-inflammatory phenotype. Impaired insulin sensitivity is accompanied by increased activation of hepatic c-JNK/SAP in Cd39/Entpd1 mice after injection of ATP in vivo. This results in decreased tyrosine phosphorylation of IRS-2 with impeded insulin signaling.

Conclusions: CD39/Entpd1 is a modulator of extracellular nucleotide signaling and also influences metabolism. Deletion of Cd39/Entpd1 both directly and indirectly impacts insulin regulation and hepatic glucose metabolism. Extracellular nucleotides serve as “metabolokines” indicating further links between inflammation and associated metabolic derangements.
Purinergic signaling elements comprise a ubiquitous sensing network within the extracellular environment. In this system, extracellular nucleotides, such as ATP, ADP and UTP, and extracellular nucleosides, such as adenosine, trigger differential cellular responses(1;2). Purinergic signaling pathways require extracellular nucleotide release mechanisms, purinergic receptors for these mediators (P2-receptors) and regulated expression of ectonucleotidases that hydrolyze extracellular nucleotides to generate adenosine(3-5). Extracellular nucleotides are provided by the secretion/release of intracellular substrates and levels are increased in hypoxia, injury, mechanical stress and inflammation.

Several reports suggest roles for extracellular nucleotides and nucleosides in glucose homeostasis; e.g., stimulation of insulin secretion from islets (6-8), modulation of glucose uptake (9;10). P2-receptors are expressed by insulin-sensitive tissues and also by immune cells. Extracellular nucleotides activate inflammatory pathways, inducing expression of proinflammatory cytokines including interleukin-1(11), interferon-gamma(12) and activating inflammatory kinase such as c-JNK through the activation of P2-receptors (13;14). Protein kinase C can be also activated by these pathways(15). These kinases phosphorylate serine residues of insulin receptor substrate-1/2(IRS-1/2), resulting in impaired insulin signaling by precluding tyrosine phosphorylation required to recruit PI-3 kinase(16;17).

CD39/ENTPD1 is an ecto-enzyme that hydrolyzes extracellular nucleotides, and is predominantly expressed in vascular endothelial cells and immune cells. We have previously shown that deletion of Cd39/Entpd1 results in disordered purinergic signaling responses that compromise vascular thromboregulation and augment inflammatory responses(3;5;12). Here we show that Cd39/Entpd1 null mice show impaired glucose tolerance secondary, at least in part, to hepatic insulin-resistance. This phenomenon is associated with increased levels of hepatocyte c-JNK activation in response to extracellular nucleotides resulting in aberrant IRS-2 phosphorylation within the livers of the mutant mice.

Our studies clearly establish links between extracellular nucleotide-mediated regulation of glucose metabolism and inflammation that are uniquely influenced by the vascular expression of Cd39/Entpd1.

MATERIALS AND METHODS

Animals. Cd39/Entpd1 null mice have been described in detail elsewhere (3) and were backcrossed 6 times onto C57BL/6 genetic background. C57BL/6 wild type (wt) mice were obtained from Taconic Co. Male mice were used in all experiments. Mice were housed under conditions of controlled temperature and illumination (12 hr light cycle, lights on at 7:00h). Unless otherwise stated, mice were fed a normal chow diet ad libitum.

All research and animal care protocols were approved by the Beth Israel Deaconess Medical Center Animal Experimentation Ethics Committee.

Glucose tolerance and insulin tolerance tests. Glucose tolerance test (GTT) was carried out with overnight-fasted (16 hr) mice at 9w and 16w old. GTT was performed by intraperitoneal injection of glucose (1g D-glucose/kg body weight). Insulin tolerance test (ITT) was performed by intraperitoneal injection of human regular insulin (0.75U insulin/kg body weight; Eli Lilly and Co.) at 2 hours after removal of food. Blood glucose levels were determined with the One Touch Ultra blood glucose monitoring system (LifeScan, Inc.). To determine effects of exogenous nucleotides on GTT, 7w old wt mice were fasted overnight. After basal plasma glucose was measured, subsequently the experimental mice were injected with ATP, ADP, UTP or UDP solution (0.5 mmoles/kg body weight) intraperitoneally whereas control animals were administered saline. At 30 minutes, each animal was subjected to GTT (n=5-6 per group). The
effects of an ecto-ATPase inhibitor ARL-67156 (5 mg/kg body weight) on GTT were examined in a similar manner.

Hyperinsulinemic euglycemic clamp study. Hyperinsulinemic euglycemic clamp studies were performed on 16-17wk old mice. Four days before experiments, an indwelling catheter was inserted in the right internal jugular vein of experimental animals. After an overnight fast, a 120-minute hyperinsulinemic-euglycemic clamp was conducted in awake mice, as previously described (18). Infusions of human insulin (Eli Lilly and Co.) at a rate of 15 pmol/kg/min raised plasma insulin levels to approximately 900 pM (WT:863.0±151.4 pM (n=8), KO:890.3±108.4 pM (n=8). p=0.8741), and 40% glucose was infused at variable rates to clamp the plasma glucose levels at approximately 6.5 mM using microdialysis pumps. At the end of the clamp studies, each tissue was harvested and frozen in liquid nitrogen. Clamp results were analyzed as described previously (18).

Measurements of metabolites, serum hormones and cytokines. Insulin concentrations were determined using a rat insulin ELISA kit with mouse insulin standards (Crystal Chem. Inc.). Serum triglyceride and FFA concentrations were measured using a colorimetric kit (Sigma-Aldrich) and a NEFA-C kit (Wako Chem.), respectively. Serum 3-hydroxybutyric acid levels were determined using a colorimetric assay kit (Wako Chem). Plasma leptin was measured using a mouse leptin ELISA kit (Crystal Chem. Inc.) and adiponectin, a mouse adiponectin RIA kit (Linco Research Inc.), respectively. Urine catecholamine levels were determined by an ELISA kit (Alpco Diagnostics). Serum cytokines levels (Interleukin-1β, Interleukin-6, Interferon-γ and Tumor Necrosis Factor-α) were measured by ELISA (eBiosciences).

Measurements of NFκB p65. Liver nuclear extracts were prepared with the Nuclear Extract Kit (Active Motif), according to the manufacture's instruction. NFκB p65 levels in equal amounts of nuclear extracts were determined with TransAM NFKBp65 (Active Motif).

Body composition. A PIXIMUS Densitometer (LUNAR Corp.) was used for the measurement of body composition in anesthetized mice (19).

Indirect calorimetry and daily activity. In vivo indirect calorimetry was performed in metabolic chambers using an Oxymax system (Columbus Instruments) in mice at 16-17w. To calculate oxygen consumption (VO₂), carbon dioxide production (VCO₂), and RQ (ratio of VCO₂ to VO₂), gas concentrations were monitored at the inlet and outlet of the sealed chambers. Global daily activity was monitored by counting the number of times that an individual mouse blocked an infrared beam running along x- and y-axes inside an individual cage.

Isolation and culture of hepatocytes. Hepatocytes were isolated from 7w old mice according to the method of Harman et al.(20). Hepatocytes were further purified by centrifugation with Percoll and plated onto gelatin-coated dishes. Unattached cells were removed at 6hr. The cells were used for experiments at 24 hr post-isolation.

Glucose uptake by cultured hepatocytes. Primary murine hepatocytes were plated onto 6 well plates at 4x10⁵ cells/well. The cells were cultured in a 37°C CO₂ incubator in William’s E buffer with 10% FCS. Subsequently, cells were serum starved overnight in William’s E buffer with 0.1% BSA. The cells were washed twice with Krebs--Ringer/Pi/Hepes (KRPH), and treated with either 100 nM insulin or KRPH buffer (control) for 30 minutes at 37°C, followed by addition of 10 mM 2-deoxy-D-glucose containing 0.3 mCi/ml 2-deoxy-D-[³H]glucose. After a 10 minute incubation, the cells were washed with ice-cold PBS three times, air-dried, then lysed with ice-cold 0.2 N NaOH. Radioactivity was determined by scintillation counting and protein concentrations were determined using the BioRad DC protein assay. 2-DG uptake was expressed as C.P.M/mg/ml of protein concentration. Similar protocol was used to measure 2-deoxy-D-
[^3]H]glucose uptake in wt cells that were either pretreated with 100 µM ATP or KRPH buffer for 30 minutes prior to commencing the insulin-stimulated 2-DG uptake assay.

**Measurement of gluconeogenesis in cultured hepatocytes.** Primary hepatocytes were serum starved overnight in DMEM with 0.4% FCS and 5mM D-glucose, washed thrice with KRPH buffer and incubated with KRPH buffer containing 20mM lactate and 2mM pyruvate (basal) or KRPH buffer containing either 10nM insulin or 100nM insulin with 20mM lactate and 2mM pyruvate. Glucose content in each aliquot was measured using Amplex Red Glucose Assay (Invitrogen Corp). Glucose production was expressed as measured fluorescence/mg/ml of protein concentration. Similar experimental protocol was used to measure glucose production in wt cells that were either pre-treated with 100 µM ATP or KRPH buffer for 30 minutes prior to commencing the insulin stimulated gluconeogenesis assay.

**Western blotting.** Tissue or cell lysates were prepared in the lysis buffer as described previously(18). Clarified supernatant was separated on SDS-PAGE under reducing condition and then transferred to PVDF membrane for Western blot analysis. For IRS-1 and IRS-2 studies, 500 µg of cleared lysate was incubated with anti-IRS-1 or anti-IRS-2 antibodies (Upstate Inc.), then captured with protein-A/G Sepharose beads (Sigma-Aldrich Co.). After washing the beads, IRS-1 or 2 was eluted in SDS-loading buffer and subjected to Western blot analysis with anti phospho-tyrosine antibody or anti IRS-1 or anti IRS-2 antibodies. Images were taken on Kodak chemiluminescent image station, 2000MM and quantified with an accompanying software program (Kodak). Detection of either phospho-JNK or JNK was carried out using anti p-JNK antibody or anti JNK antibody (Cell Signaling Technolgies). Phospho-Ser731-IRS-2 was detected using specific antibodies purchased from Abcam Inc.

**Administration of ATP into portal vein.** Twelve weeks old male mice were anesthetized with Nembutal, then 10mM ATP, in PBS was administered via a portal vein at 1.4 µl/g and at 2.5 sec/g body weight using a microinfusion pump. At 30 min of infusion, mice were sacrificed by exsanguination and immediately the perfused liver was snap-frozen in liquid nitrogen.

**Immunohistochemistry.** Animals were anesthetized with Avertin and sacrificed by exsanguination. Pancreatic, hepatic and skeletal muscle tissues were immediately embedded in OCT compound in isopentene-liquid nitrogen freezing bath. Brown adipose tissue was fixed in zinc-fixative solution (BD Biosciences) for 1.5 days. The tissue sections were used for immunohistochemical staining with antibodies against Cd39/Entpd1(3), Cd31 (BD Biosciences), insulin and glucagon (DAKO).

**Real-Time PCR for gluconeogenetic enzymes.** Liver total RNA was purified with Qiagen RNAeasy mini-kit and used to synthesis cDNA using Invitrogen first strand cDNA synthesis kit. Real-Time PCR was performed with iTaq SYBR Green Supermix with ROX (Bio-rad) on Stratagene MX3000P quantitative PCR system. The primers used were: TGGTAGCCCTGTCTTTCTTTG and TTCCAGCATTACACTTTCCT for Glucose-6-phosphatase (G6P), ACACACACATGCTCACAC and ATCACCGCATAGTCTCTGAA for Phosphoenolpyruvate Carboxylase (PEPCK) (21). Actin values were used for normalization.

**RESULTS**

**Cd39/Entpd1 deletion results in abnormalities in glucose and insulin tolerance tests.** Cd39/Entpd1 knockout (Cd39/Entpd1−/−) mice were subjected to GTT and ITT at 9w of age (Fig. 1). Blood glucose levels were significantly higher in Cd39/Entpd1−/− mice throughout the GTT when compared to wt mice (Fig. 1A). Plasma insulin levels during GTT were also consistently higher in Cd39/Entpd1−/− mice when contrasted to wt mice, suggesting insulin-resistance in Cd39/Entpd1−/− mice (Fig.
Cd39/Entpd1^−/− mice also exhibited impaired responses to insulin during ITT (Fig. 1C). Cd39/Entpd1^−/− mice at 16w likewise exhibited glucose intolerance and insulin resistance as determined by GTT and ITT. There were no differences in fasting and fed state blood glucose levels between Cd39/Entpd1^−/− and wt mice.

We then examined effects of exogenous nucleotide administration upon GTT in wt mice. Increases in blood glucose levels were observed at 30 min after administration of ATP vs. saline (Fig. 1D). These plasma glucose increments were sustained in the group treated with ATP during GTT. The pre-administration of other nucleotides, UTP, ADP, and UDP did not substantially affect glucose tolerance in wt mice (Figs. 1D and 1E). Pre-administration of ARL-67156, a competitive ecto-ATPase inhibitor, resulted in increases in glucose levels basally and following GTT in wt mice (Fig. 1F).

**Metabolic Parameters.** Insulin-resistance is often accompanied with obesity and disordered lipid metabolism. We therefore assessed other parameters relevant to these disorder of metabolism (Table 1). Cd39/Entpd1^−/− mice are clearly not obese. Plasma insulin levels were significantly higher in fed Cd39/Entpd1^−/− mice at all ages tested, and in fasting Cd39/Entpd1^−/− mice at 16 weeks of age. Plasma free fatty acid and triglyceride levels in Cd39/Entpd1^−/− mice were not generally different from wt mice but became statistically higher at 35 weeks of age. Plasma leptin levels of Cd39/Entpd1^−/− mice were not generally different from wt mice but became statistically higher at 35 weeks of age. Plasma leptin levels of Cd39/Entpd1^−/− mice were significantly higher than in wt mice but adiponectin levels were comparable. Plasma 3-hydroxybutyrate levels were comparable in Cd39/Entpd1^−/− mice and wt mice.

We also examined sympathetic reactivity in Cd39/Entpd1^−/− mice. Total adrenaline and noradrenalin levels in urine were not statistically different between these two groups (Adrenaline; Cd39/Entpd1^−/− mice: 1.49±0.50 pg/day vs wt mice: 1.64±0.48 pg/day, p=0.83. Noradrenaline; Cd39/Entpd1^−/− mice: 2.76±0.90 pg/day vs wt mice: 3.06±0.73 pg/day, p=0.79.).

**Energy expenditure and activity.** We examined the impact of Cd39/Entpd1 deletion on energy metabolism (Fig. 2). Heat generation (Fig. 2A), oxygen consumption (Fig. 2B) and respiratory ratio (Fig. 2C) were significantly lower in Cd39/Entpd1^−/− mice both during dark and light periods. However, food intake and daily activities of Cd39/Entpd1^−/− mice were comparable to those of wt mice (Fig. 2D).

**Hyperinsulinemic euglycemic clamp studies.** In order to analyze systemic glucose uptake and usage, we performed hyperinsulinemic euglycemic clamp studies. Glucose uptakes in the extrahepatic tissues were not statistically different between Cd39/Entpd1^−/− and wt mice (Fig. 3A). Glucose infusion rates (GINF) during glucose clamp, were however significantly lower in Cd39/Entpd1^−/− mice (Fig. 3B). Coincidently, glucose disappearance rates (Rd) were lower in Cd39/Entpd1^−/− mice (Fig. 3C). Basal hepatic glucose production (HGP) was normal but the extent of insulin-dependent suppression of HGP was significantly decreased in Cd39/Entpd1^−/− mice (Fig. 3D; Cd39/Entpd1^−/− mice: 26.6±14.4 % vs wt mice: 69.5±9.6 %, p=0.021). These results suggest that systemic insulin resistance and glucose intolerance of Cd39/Entpd1^−/− mice were associated with impaired hepatic responses to insulin and decreased Rd.

PEPCK and G6P expressions by RT-PCR were significantly higher in fasting Cd39/Entpd1^−/− mice liver than in wt mice liver (PEPCK; Cd39/Entpd1^−/− mice: 128.8±10.2 % vs wt mice: 100.0±6.0 %, p=0.027. G6P; Cd39/Entpd1^−/− mice: 240.6±26.1 % vs wt mice: 100.0±7.3 %, p=0.001).

**Localization of Cd39/Entpd1 in mouse liver, pancreas, brown adipose tissue and skeletal muscles.** High levels of Cd39/Entpd1 staining were observed in the endothelium with same expression in vascular smooth muscle cells of muscularized blood vessels within liver and skeletal muscle.
Hepatic sinusoidal endothelial cells do not express Cd39/Entpd1 under basal conditions; however, Cd39/Entpd1 is expressed by certain hepatic sinusoidal cells e.g. Kupffer cells and NKT cells (22;23).

Hepatocytes and skeletal muscle cells were negative for Cd39/Entpd1 (Fig. 4, A and B). In brown adipose tissue (BAT), Cd39/Entpd1 expression was likewise restricted to vascular endothelium of veins and arterioles but not noted in the microvasculature (Fig. 4, C). In pancreas, the expression was also detected only in the vascular endothelial cells and vascular smooth muscle cells (Fig.4, D and I). In islets of Langerhans of the pancreas, the beta-cells, alpha-cells and delta-cells were all negative for Cd39/Entpd1. However the microvasculature endothelial cells did stain for Cd39/Entpd1 in the pancreatic tissues and islets. Both wt and Cd39/Entpd1-/- islets stained for glucagon in a comparable manner (Fig. 4, K and O); Cd39/Entpd1-/- islets reacted strongly for insulin (Fig. 4, L and P).

**Effect of extracellular ATP on glucose metabolism in hepatocytes.** We next analyzed glucose metabolism in cultured primary hepatocytes isolated from Cd39/Entpd1-/- mice as hyperinsulinemic euglycemic clamp studies had suggested impaired hepatic glucose homeostasis in Cd39/Entpd1-/- mice. Overall glucose uptakes were significantly decreased in Cd39/Entpd1-/- hepatocytes in vitro (Fig. 5A).

The glucose uptake by wt hepatocytes was significantly decreased in the presence of ATP (Fig. 5B). Major contributions of liver to systemic glucose homeostasis involve regulation of glucose production that is suppressed by insulin. Glucose production was much higher in Cd39/Entpd1-/- hepatocytes than the wt hepatocytes. The increased level of glucose production by Cd39/Entpd1-/- hepatocytes was not significantly suppressed by insulin. In contrast, wt hepatocytes had the expected statistically significant suppression by insulin with respect to the glucose production (Fig. 5C). Exogenous extracellular ATP induced also higher levels of glucose production in wt hepatocytes; this was not suppressed by insulin (Fig. 5D).

**Susceptibility of c-JNK phosphorylation by exogenous ATP treatment.** c-JNK is a member of MAP kinase family that is activated by various pro-inflammatory cytokines. The activated c-JNK phosphorylates serine residues of IRS-1/2 and consequently prevents tyrosine phosphorylation of IRS-1/2 by insulin receptor(16). As extracellular ATP treatment induces c-JNK activation in rat hepatocytes(14), we examined phosphorylation levels of c-JNK in mouse liver after portal venous administration of ATP in vivo. We noted that phosphorylation of c-JNK in Cd39/Entpd1-/- mice liver at 30 min post ATP administration was significantly higher than in wt mice (Fig. 6A).

**Decreased tyrosine phosphorylation of hepatic IRS-2 in Cd39/Entpd1-/- mice.** Tyrosine phosphorylation of IRS could be hampered by phosphorylation of adjacent serine residues of IRS mediated by inflammatory kinase such as c-JNK or protein kinase C (PKC) (16;17). Such kinases are activated through the activation of purinergic receptors by extracellular ATP (14;15). We therefore examined phosphorylation of IRS-1 and -2 in wt and Cd39/Entpd1-/- mice liver to dissect out differential responses to insulin in vivo. There were no differences in levels of tyrosine phosphorylation of IRS-1 between wt and Cd39/Entpd1-/- mice liver samples after insulin administration (Fig. 7). However, tyrosine phosphorylation of IRS-2 was markedly suppressed in Cd39/Entpd1-/- murine liver relatively to wt samples post-injection of insulin. Relative levels of hepatic Ser-731 phosphorylation of IRS-2 were higher in Cd39/Entpd1-/- mice when contrasted to wt mice tissues (Fig. 7D).

**Serum proinflammatory cytokine levels.** Increased levels of proinflammatory cytokines are known to induce insulin-resistance by activating kinases such as c-JNK. Extracellular nucleotides also clearly activate inflammatory pathways. Therefore we have measured the serum levels of
proinflammatory cytokines as surrogates for basal inflammatory responses in Cd39/Entpd1−/− mice. IL-6, IFN-γ, IL-1β and TNF-α levels are significantly higher in Cd39/Entpd1−/− mice than in wt mice (Table 1). NFKBp65 levels were also higher in the Cd39/Entpd1−/− mice liver treated with exogenous ATP (Cd39/Entpd1−/− mice:100.0±9.5 vs wt mice:178.5±24.1 (Arbitrary units) p=0.0019).

**DISCUSSION**

We have shown that deletion of Cd39/Entpd1 results in abnormalities of glucose homeostasis and causes insulin-resistance. We show that the plasma insulin levels are higher in Cd39/Entpd1−/− mice than wt mice during GTT and islets stain strongly for insulin in Cd39/Entpd1−/− mice tissues. Glucose intolerance in Cd39/Entpd1−/− mice does not appear to be related to an intrinsic abnormality in insulin secretion from beta-cells. Significant effects of ATP administration on the GTT in contrast to other nucleotides suggest involvement of P2X receptors that are uniquely activated by ATP in the expression of glucose intolerance.

Cd39/Entpd1−/− mice are not obese but leptin levels are increased. Leptin is an important anorexigenic hormone secreted from adipocytes(24-26). Insulin may induce secretion of leptin(27). Therefore it is possible that the hyperinsulinemia of Cd39/Entpd1−/− mice might be a cause for hyperleptinemia. The food intake of Cd39/Entpd1−/− mice was comparable to those of wt mice despite the increased levels of plasma leptin. Leptin is also known to increase energy expenditure. However, calorimetric analysis of Cd39/Entpd1−/− mice revealed decreased heat generation and energy consumption both during dark and light cycles. Respiratory ratios of Cd39/Entpd1−/− mice were slightly lower than wt mice. Potentially other tissue specific mechanisms might exist for the regulation of energy consumption by extracellular nucleotides. Our current data however suggest the possibility of both leptin-resistance as well as insulin-resistance in Cd39/Entpd1−/− mice.

Hyperinsulinemic euglycemic clamp studies identified the liver as a major target organ for the effects of Cd39/Entpd1 on glucose homeostasis and insulin-resistance. Immunohistochemical studies showing Cd39/Entpd1 to be expressed dominantly within the vasculature, suggest that observed insulin resistance are secondary to modulation of extracellular and plasma nucleotides levels brought by loss of vascular ecto-nucleotidase activity. This is different mechanistically from the type of insulin-resistance induced by a K121Q variant of PC-1 (ecto-nucleotide pyrophosphatase/phosphodiesterase). The latter requires direct/physical interactions between insulin-receptor and PC-1(29;30). Furthermore, PC-1 is expressed in skeletal muscle cells and hepatocytes unlike the unique vascular expression of Cd39/Entpd1.

We next examined aspects of glucose metabolism in primary hepatocyte cultures of Cd39/Entpd1−/− and wt mice. In general, glucose transport by hepatocytes is insulin-independent. However, expression of glucokinase is induced by insulin. We noted that the overall uptake of glucose was significantly decreased in Cd39/Entpd1−/− mice hepatocytes. Glucose uptake by wt primary culture hepatocyte was also significantly decreased by treatment with extracellular ATP, albeit not to the same extent as with Cd39/Entpd1−/− hepatocytes. ATP is known to induce hepatic gluconeogenesis(28) and impact upon glucose release via P2X4 receptor mediated increases in glycogenolysis (29). Our and these published data suggest that the cellular phenotype seen in the Cd39/Entpd1−/− mice may be directly dependent upon exposure to extracellular nucleotides. We suggest that these are differential and persistent purinergic responses in these target tissues that are impacted upon by loss of Cd39/Entpd1 in vasculature. We infer that the reduced Rd values observed in Cd39/Entpd1−/− mice during the hyperinsulinemic euglycemic studies are secondary, in substantial part, to the decreased glucose uptake in hepatocytes.
Other contributions of insulin to hepatic glucose homeostasis include the suppression of glucose production. Liver specific insulin receptor knockout mice exhibit hepatic insulin resistance i.e. impaired insulin mediated suppression of hepatic glucose production that is accompanied by elevated plasma insulin levels. These features are also observed in Cd39/Entpd1<sup>−/−</sup> mice suggesting indirect effects on insulin receptor activity(30).

Cd39/Entpd1<sup>−/−</sup> mice hepatocytes also show significantly higher basal glucose production than seen in wt mice hepatocytes. Furthermore, the glucose production of Cd39/Entpd1<sup>−/−</sup> mice hepatocytes is not suppressed by the treatment of insulin unlike in the instance of wt mice hepatocytes where this is suppressed by insulin. Interestingly, ATP treatment of wt hepatocytes also induces heightened hepatic glucose production, even in the presence of insulin. These results confirm that extracellular ATP regulates glucose homeostasis in hepatocytes. Furthermore, Cd39/Entpd1 expressed on adjacent cells such as endothelial cells, is likely to regulate insulin signals in the hepatocyte by modulating extracellular nucleotide levels in the immediate microenvironment in vivo. It is worthy of mention that vascular endothelial specific insulin receptor knockout mice do not exhibit insulin resistance (31). We propose that insulin resistance observed in Cd39/Entpd1<sup>−/−</sup> mice is not directly caused by modulation of vasculature functions but involves other factors, e.g. paracrine fluxes of extracellular nucleotides that impact target tissues such as the hepatocyte.

Activation of cellular signaling cascades by proinflammatory cytokines such as TNFα and IL-1, free fatty acids or nucleotides(14), can also induce cellular activation via inflammatory kinases. Activation of inflammatory kinases such as c-JNK, also causes aberrant serine phosphorylation of IRS. This specific form of serine phosphorylation prevents subsequent tyrosine phosphorylation by insulin receptors and association of PI3 kinases, leading to impaired insulin signal transduction(32;33).

To analyze the molecular mechanisms for insulin resistance of Cd39/Entpd1<sup>−/−</sup> mice, we analyzed tyrosine phosphorylation of IRS-1 and -2. Insulin-stimulated tyrosine phosphorylation of IRS-2 was significantly decreased in Cd39/Entpd1<sup>−/−</sup> mice liver. In addition, phosphorylation of c-JNK induced by administration of ATP was also significantly increased in Cd39/Entpd1<sup>−/−</sup> mice liver. These data suggest that insulin resistance of Cd39/Entpd1<sup>−/−</sup> mice is associated with increased activation of hepatic c-JNK/SAP by extracellular ATP. This likely results in aberrant serine phosphorylation and consequently decreased tyrosine phosphorylation of IRS-2. Similar patterns of aberrant IRS-2 phosphorylation have been noted in other insulin-resistance states (34).

Furthermore, several features observed in Cd39/Entpd1<sup>−/−</sup> mice are also seen in IRS-2<sup>−/−</sup> mice: both show elevated plasma insulin levels and have normal fasting blood glucose levels with impaired glucose homeostasis in the liver. IRS-2<sup>−/−</sup> mice however show increases in blood glucose levels with advancing age due to lack of compensatory beta-cell hyperplasia (35).

Other cellular candidates for mediating insulin resistance of Cd39/Entpd1<sup>−/−</sup> mice include immune cells such as macrophages in adipose tissues and the Kupffer cells of the liver, as alluded to above (35). Activation of purinergic signaling pathways is already known to induce secretion of cytokines from both immune and endothelial cells (36-40). We show that serum proinflammatory cytokine levels (IL-1β, IFN-γ, IL-6 and TNFα) are significantly higher in Cd39/Entpd1<sup>−/−</sup> mice than wt mice. Accordingly, hypersecretion of select cytokines by monocytes and other cells also might contribute, at least in part, to the development of insulin-resistance in Cd39/Entpd1<sup>−/−</sup> mice.

Our data suggest that insulin resistance in Cd39/NTPDase1<sup>−/−</sup> mice is associated with disordered extracellular nucleotide-signaling that directly impact
important metabolic pathways in hepatocytes via aberrant IRS-2 phosphorylation. However, we do not exclude the possibility that deletion of Cd39 might also contribute to insulin resistance in a somewhat more indirect manner via the induction of inflammatory cytokines. The relative contributions of these two non-exclusive mechanisms remain to be determined.

We have already reported on other vascular and immune abnormalities in Cd39/Entpd1−/− mice. This described phenotype includes disordered thromboregulation, aberrant inflammatory responses, impaired angiogenesis and regeneration of the liver, impaired function of regulatory T-cells, predisposition to diabetic renal diseases with increased proteinuria and hypertension (3;5;12;15;40). To these manifestations of Cd39/Entpd1 deletion, we now add metabolic consequences.

In conclusion, Cd39/Entpd1 expressed by the vasculature and by immune cells, serves as an important modulator of hepatic carbohydrate metabolism. The pathogenetic mechanism involves failure to controlling extracellular nucleotide fluxes that both directly and indirectly impact insulin responsiveness.

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### Table 1. Comparison of Blood analysis data of Cd39/Entpd1−/−

|                     | Wild Type | Cd39/Entpd1−/− | P     |
|---------------------|-----------|----------------|-------|
| **Body Weight (g)** |           |                |       |
| 9 w (fasting)       | 24.6±0.3  | 25.3±0.9       | 0.426 |
| 9 w (fed)           | 26.6±0.4  | 27.3±0.9       | 0.418 |
| 16 w (fasting)      | 27.2±0.3  | 29.1±1.1       | 0.11  |
| 16 w (fed)          | 30.2±0.4  | 31.6±1.0       | 0.24  |
| **Fasting Glucose (mg/dl)** |         |                |       |
| 9 w                 | 95±5      | 97±6           | 0.426 |
| 16 w                | 105±3     | 104±2          | 0.331 |
| **Fed Glucose (mg/dl)** |        |                |       |
| 9 w                 | 185±8     | 190±6          | 0.418 |
| 16 w                | 189±3.7   | 202±8.1        | 0.09  |
| **Fasting Insulin (pg/ml)** |        |                |       |
| 9 w                 | 0.34±0.07 | 0.38±0.13      | 0.791 |
| 16 w                | 0.39±0.12 | 0.79±0.18      | 0.028 |
| **Fed Insulin (pg/ml)** |        |                |       |
| 9 w                 | 1.51±0.07 | 2.02±0.22      | 0.0366|
| 16 w                | 0.74±0.05 | 1.74±0.24      | 0.005 |
| **Fasting Triglycerides (mg/dl)** |     |                |       |
| 9 w                 | 35±9      | 47±4           | 0.252 |
| 15 w                | 60±4      | 65±5           | 0.225 |
| 35 w                | 45±4      | 95±15          | 0.01  |
| **Fed Triglycerides (mg/dl)** |        |                |       |
| 9 w                 | 57±6      | 57±4           | 0.976 |
| 16 w                | 40±5      | 54±5           | 0.067 |
| **Fasting FFA (mM)** |          |                |       |
| 9 w                 | 1.08±0.07 | 0.98±0.04      | 0.261 |
| 15 w                | 1.21±0.05 | 1.17±0.03      | 0.492 |
| 35 w                | 0.71±0.04 | 1.01±0.11      | 0.013 |
| **Fed FFA (mM)**    |          |                |       |
| 9 w                 | 0.49±0.02 | 0.53±0.02      | 0.135 |
| 16 w                | 2.94±0.08 | 3.14±0.13      | 0.212 |
| **Fasting 3-Hydroxybutyrate (mM)** |    |                |       |
| 15 w                | 1.58±0.05 | 1.64±0.05      | 0.453 |
| **Leptin (ng/ml)**  |          |                |       |
| 16 w                | 12.1±0.8  | 18.6±2.8       | 0.036 |
| **Adiponectin (µg/ml)** |         |                |       |
| 16 w                | 9.1±0.6   | 8.7±0.4        | 0.623 |
| **Visceral Fat (% of BW)** |      |                |       |
| 16 w                | 25.3±0.7  | 27.2±1.9       | 0.352 |
| **Total Fat (%)**   |          |                |       |
| 16 w                | 24.6±0.5  | 27.0±1.9       | 0.131 |
| **Serum cytokines (15w, Fasting)** |    |                |       |
| IL-1β (pg/ml)       | 1.16±0.88 | 8.05±3.43      | 0.049 |
| IFN-γ (pg/ml)       | 41.5±6.2  | 80.6±10.5      | 0.01  |
| IL-6 (Pg/ml)        | 1.71±0.75 | 5.14±1.61      | 0.037 |
| TNF-α (Arbitrary Units) | N.D (below range) | 1.53±0.45 | <0.05 |

Data are mean ± S.E from six to 10 animals of each group.
FIGURE LEGENDS

Figure 1. Cd39/Entpd1^-/- mice exhibit impaired glucose tolerance and insulin resistance. A, Glucose tolerance test. B, Plasma insulin levels during GTT. C, Insulin tolerance test. Closed square, Cd39/Entpd1^-/- mice. Open square, wt mice. (9w old, n=6). D-F, Effects of different nucleotides on glucose tolerance test in wt mice (n=5-6). D, Open square, ATP-treated mice. Closed triangle, UTP-treated mice. Closed square, saline control. E, Open square, ADP-treated mice. Closed square, UDP-treated mice. Closed square, saline control. F, Open-triangle, ARL-67156-treated mice. Closed square, saline control. Data are mean ± s.e. **, p < 0.05. *, p<0.1.

Figure 2. Calorimetric and functional analyses of Cd39/Entpd1^-/- mice. Cd39/Entpd1^-/- mice showed decreased energy expenditure. Food intake did not differ between Cd39/Entpd1^-/- and wt mice. A, Oxygen consumption. B, Heat generation. C, Respiratory ratio. D, Accumulated food intake over 3 days. Black bar, Cd39/Entpd1^-/- mice. Open bar, wt mice. (n=6 for wt, 7 for Cd39/Entpd1^-/- mice). Results were analyzed using data from three consecutive days. Data are mean ± s.e. **, p < 0.05.

Figure 3. Cd39/Entpd1^-/- mice manifest impaired hepatic glucose homeostasis during hyperinsulinemic euglycemic clamp. A, Tissue glucose uptake estimated by 2-DG. B, Glucose infusion rate (GINF). C, Glucose disappearance rate (Rd). D, Hepatic glucose production (HGP) and its suppression by insulin. A-C, Black bar, Cd39/Entpd1^-/- mice. Open bar, wt mice. D. Black bar, Cd39/Entpd1^-/- mice. Open bar, wt mice. Solid bar, basal. Hatched bar, with insulin (n=6 for wt, 7 for Cd39/Entpd1^-/- mice). Data are mean ± s.e. **, p < 0.05.

Figure 4. Immunohistochemical analysis. Various insulin sensitive tissues together with pancreatic islets were analyzed for Cd39 expression and are compared with the vascular endothelial marker Cd31. In lower right panels, islet content of glucagon and insulin are also displayed.

A - D, I and M, show expression of Cd39/Entpd1. E - H, J and N, are stained for Cd31.
A and E, are from wt Liver. B and F, wt skeletal muscle. C and G, wt brown adipose tissue. Inserted microimage, at large magnification. (+/+), wt BAT. (-/-) in C, Cd39/Entpd1 null BAT serves as a negative control. D and H, wt pancreas, respectively. I and J are islet tissues from wt whereas M and N are from the Cd39/Entpd1 null mice.
K (wt) and O (null), are stained for glucagon. L (wt) and P (null), show the staining patterns for insulin (see text for details).

Figure 5. Effect of extracellular ATP on glucose metabolism in hepatocytes. A, 2-DG uptake by Cd39/Entpd1^-/- mice and wt mice hepatocytes. B, Effect of extracellular ATP on 2-DG uptake by wt mice hepatocytes. C, Hepatic glucose production of Cd39/Entpd1^-/- mice and wt mice hepatocytes and its suppression by insulin. D, Effect of Extracellular ATP on glucose production in wt hepatocytes. Experiment was performed in triplicates. A and C, Black bar, Cd39/Entpd1^-/- mice. Open bar, wt mice. B and D, Open bar, without ATP. Black bar, with ATP. **, p < 0.05. *, P>0.05 by ANOVA but p<0.05 by Student’s t-test.
**Figure 6.** c-JNK phosphorylation in murine liver. Mice were administered ATP via portal vein and the liver was harvested at 30 min after injection. The lysates were analyzed by Western blotting with anti-phospho-JNK antibodies or anti-JNK antibodies. A, Quantification of p-JNK to total JNK ratio by Western blot analysis. Open bar, wt. Black bar, Cd39/Entpd1<sup>−/−</sup>. Solid bar, saline control mice. Hatched bar, ATP-treated mice (n=3). B, Representative staining of Western blot for p-JNK and JNK. P, a positive control. C, Quantification of NFκBp65 in murine liver nuclear extracts (n=8-9). Hatched Bar (black on white), wt mice. Hatched Bar (white on black), Cd39/Entpd1<sup>−/−</sup> mice. **, p < 0.05. *, P>0.05 by ANOVA but p<0.05 by Student’s t-test

**Figure 7.** Impaired tyrosine phosphorylation of IRS-2 in Cd39/Entpd1<sup>−/−</sup> liver. Age-matched 12-15w of male mice were starved for 16 hr (17:00h-10:00h), then received human regular insulin at 5 U/kg body weight, i.v. and the tissues were collected at 5 min after injection. Lysates were immunoprecipitated with anti-IRS antibodies and protein A/G sepharose and then probed with anti-tyrosine antibodies. A, Quantification of p-IRS-1/2 to total IRS-1/2 by Western blot analysis. B, Representative staining of Western blot for p-IRS-1/2 or total IRS-1/2. Open bar, wt mice. Black Bar, Cd39/Entpd1<sup>−/−</sup> mice. Solid bar, saline treated. Hatched bar, insulin-treated. (n=3). **, p < 0.05. C, Quantification of p-Ser731-IRS-2 to total IRS-2 by Western blot analysis. D, Representative staining of Western blot for p-Ser731-IRS-2 or total IRS-2. Open bar, wt mice. Black Bar, Cd39/Entpd1<sup>−/−</sup> mice. (n=3). **, p < 0.05.
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Figure 4

Tissues

| Tissues      | liver | muscle | BAT | pancreas |
|--------------|-------|--------|-----|----------|
| Cd39/Entpd1  |       |        |     |          |
| cd31         |       |        |     |          |
| Pancreatic islets |       |        |     |          |
| wt           |       |        |     |          |
| Cd39/Entpd1<sup>−/−</sup> |       |        |     |          |

Figure 4
Insulin resistance and Cd39

Figure 5

A. 2-DG uptake

B. Effect of ATP on 2-DG uptake

C. Glucose production

D. Effect of ATP on Glucose production

Figure 6

A

B

C

NFKBp65 (Arbitrary Units)
Insulin resistance and Cd39

Figure 7.

A. 

B. 

C. 

D. 

Figure 7.