I prostanoid receptor-mediated inflammatory pathway promotes hepatic gluconeogenesis through activation of PKA and inhibition of AKT

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Word count: 4509
Number of Figures: 8
List of Abbreviations

COX-1:cyclooxygenase-1;  COX-2:cyclooxygenase-2;  PGI_2:prostaglandin;  IP: prostaglandin receptor;  HFD:high fat diet;  cAMP:cyclic adenosine monophosphate CREB:cAMP response element-binding protein;  G6Pase:glucose-6-phosphatase; PEPCK: phosphoenolpyruvate carboxykinase;  STZ:streptozotocin;  PKC_ζ:protein kinase C zeta;  ITT:insulin tolerance test;  GTT:glucose tolerance test;  PTT:pyruvate tolerance test;  HGP: hepatocytes glucose production.
Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin (ASA), improve glucose metabolism in diabetic subjects, although the underlying mechanisms remain unclear. In this study, we observed dysregulated expression of COX-2, prostacyclin biosynthesis, and the I prostanoid receptor (IP) in the liver's response to diabetic stresses. High doses of ASA reduced hepatic prostaglandin generation and suppressed hepatic gluconeogenesis in mice during fasting, and the hypoglycemic effect of ASA could be restored by IP agonist treatment. IP deficiency inhibited starving-induced hepatic gluconeogenesis, thus inhibiting the progression of diabetes, while hepatic overexpression of IP increased gluconeogenesis. IP deletion depressed cAMP-dependent CREB phosphorylation and elevated AKT phosphorylation by suppressing PI3Kγ/PKCζ mediated TRB3 expression, which subsequently down-regulated the gluconeogenic genes for glucose-6-phosphatase (G6Pase) and phosphoenol pyruvate carboxykinase 1 (PEPCK1) in hepatocytes. We therefore conclude that suppression of IP modulation of hepatic gluconeogenesis through the PKA/CREB and PI3Kγ/PKCζ/TRB3/AKT pathways contributes to the effects of NSAIDs in diabetes.
Introduction

Glucose is the major source of energy required by most mammalian cells to maintain normal physiological functions. Glucose homeostasis is tightly regulated within a relatively narrow range by hormones such as glucagon and insulin, through balancing of glucose output by the liver and its utilization by peripheral tissues like skeletal muscle, heart and adipocytes. Liver is the dominant organ in the maintenance of glucose homeostasis, which is regulated by way of glucose production through glycogenolysis and gluconeogenesis, and glucose uptake by glycogenesis and glycolytic conversion to pyruvate (1). Circulating insulin increases in response to feeding, leading to glycogenesis and lipogenesis and the suppression of hepatic glucose production. Conversely, during fasting conditions or in the case of untreated type 1 diabetes, insulin secretion drops and glucagon secretion rises, prompting hepatic glycogenolysis and gluconeogenesis. The key regulatory enzymes for hepatic gluconeogenesis include glucose 6 phosphatase (G6Pase), fructose-1, 6-bisphosphatase and phosphoenol pyruvate carboxykinase (PEPCK, also known as PCK1). However, in patients with type 2 diabetes, the rate of hepatic gluconeogenesis is considerably elevated, contributing to both fasting hyperglycemia and exaggerated post-prandial hyperglycemia.

Prostaglandins (PGs) play important roles in inflammation-mediated diseases including diabetes mellitus (2). Elevated PGs have been observed in both type 1 and
type 2 diabetes mellitus (3,4). Epidemiological studies have indicated that the use of non-selective NSAIDs including ASA is associated with a significant reduction in the risk of diabetes in healthy populations (5). Selective COX-2 inhibitors have also been reported to increase insulin sensitivity in healthy individuals (6), and to ameliorate diabetes in experimental animals (7). These observations strongly suggest that COX-derived PGs are involved in the pathogenesis of diabetes mellitus. Moreover, clinical trials have revealed that treatment with ASA (8) results in the reduction of fasting plasma glucose and improves insulin sensitivity, while high doses of selective COX-2 inhibitors have been reported to cause hypoglycemia (9) and increase the hypoglycemic effect of oral anti-diabetic drugs (10). These observations raise the possibility that PGs play a role in carbohydrate metabolism, especially in hepatic gluconeogenesis, the predominant source of increased hepatic glucose in type 2 diabetes mellitus.

Prostanoids in liver are produced by both parenchymal hepatocytes (11) and non-hepatocyte cells such as Kupffer cells (12), and their biosynthesis and release can be regulated in response to a range of (patho) physiological stimuli to modulate hepatocyte function. In isolated rodent livers, infusion of PGF2α but not thromboxane A2 (TxA2) stimulates gluconeogenesis and glycogenolysis, and PGD2 induces hepatic glycogenolysis (13), while PGE2 inhibits glucagon-mediated gluconeogenesis from lactate (14). However, the relevance of these infusion
experiments to the autocoidal role and concentrations of endogenous eicosanoids is unclear, and the potential importance of PGI\textsubscript{2} in regulating glucose metabolism in liver is unknown.

In this study, we observed upregulation of the COX-2/PGI\textsubscript{2}/IP axis in the livers of fasted, or high-fat diet (HFD)-treated mice, or mice in which diabetes had been induced genetically or pharmacologically. Deletion of the IP conferred protection against diabetes in mice due to the suppression of hepatic gluconeogenesis. Conversely, re-expression of IP in liver, augmented hepatic glucose output and led to insulin resistance by enhancing intracellular adenylate cyclase activity and upregulating TRB3-dependent AKT phosphorylation, and subsequently promoting transcription of the key hepatic gluconeogenic enzymes PCK1 and G6Pase. These data suggest that PGI\textsubscript{2} is involved in modulation of hepatic gluconeogenesis through the IP.
Research Design and Methods

Mice

All the mice used in this study were maintained at C57BL/6 background. For the diet-induced obese model, mice were fed either a regular chow diet (SLRC, Shanghai, People’s Republic of China) or HFD (60% fat, D12492, Research Diets, New Brunswick, NJ) ad libitum for 16 weeks. For aspirin treatment, 7-week-old Ob/Ob mice received either normal drinking water or drinking water containing 600 mg/L (high-dose) aspirin, respectively, which was replaced every other day. All procedures were approved by the Institutional Animal Care and Use Committee of the Institution for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China.

Metabolic Studies

ITTs, GTTs, and PTTs were performed by i.p. injection of 0.8 U/kg insulin after 6 h fasting or 1.5g/kg glucose after overnight fasting or 2g/kg sodium pyruvate after overnight fasting, respectively. Glucose clearance was evaluated by calculating the areas under curve (AUC) as previously described (15). Glucagon challenge test was performed by i.p. injection of glucagon (150 µg/kg) after a 15-h fast (16). Measurements of oxygen consumption (VO$_2$) and respiration quotient (RER) (VCO$_2$/VO$_2$) were acquired using indirect calorimetry, as previously described (17). Murine plasma samples collected at 8-9 am were subjected to insulin and glucagon
quantitation using the Mercodia Ultrasensitive Rat Insulin ELISA kit and Glucagon ELISA kit (ALPCO Diagnostic, Salem, NH), respectively. Serum triglyceride (TG), total cholesterol (CHO), high density lipoprotein cholesterol (HDLC), and low density lipoprotein protein (LDLC) were measured using assay kits (BJKT, Beijing, People’s Republic of China). Tissue triglyceride and total cholesterol were measured using assay kits (BJKT, Beijing, People’s Republic of China) as previously described (18).

**Western Blotting**

Proteins from total cell lysates were separated by SDS-PAGE and probed with different primary antibodies against AKT (ser473), AKT (ser308), AKT, IRS (Ser307), IRS, FoxO1 (Ser253), FoxO1, GSK3β (ser9), GSK3β, Phospho-p44/42 MAPK (Thr202/Tyr204), p44/42 MAP Kinase, PDK1 and CTMP (Cell Signaling Technology, Danvers, MA); COX-1 and COX-2 (Cayman Chemical Company, Ann Arbor, MI); G6Pase and TRB3 (Santa Cruz Biotechnology, Santa Cruz, CA); PCK1 (Abcam); and β-actin (Sigma-Aldrich, St. Louis, MO); HK2 and PPAR-α (Proteintech, Wuhan, People’s Republic of China).

**Primary hepatocyte glucose production**

Primary hepatocytes were washed three times with PBS, then changed to glucose- and phenol-free DMEM with 20 mM sodium lactate and 1mM sodium pyruvate for 6 h. Glucose levels in the culture were determined using Glucose Assay kit (Sigma-Aldrich, St. Louis, USA). Total protein was used for normalization. For
adenovirus experiments, cells were infected with various adenoviruses, and HGP were quantitated after 48 h.

**Cell culture and treatments**

Primary hepatocytes were cultured in DMEM with 25 mmol/L glucose, 10% FBS, 50 µg/mL penicillin and streptomycin at 37°C, and 5% CO₂/95% air. Insulin (100 nM) from Sigma-Aldrich, glucagon (100 nM), Cicaprost (1 µM), LY294002 (25 µM) and RO32-0432 (10 µM), CAY-10441 (1 µM) from Cayman Chemical and Akt inhibitor IV (1 µM) from Calbiochem were used to treat hepatocytes as indicated.

**Determination of cellular cAMP levels**

Cellular cAMP levels were measured as previously described (19).

**In Vivo insulin signaling Assay**

For measurement of insulin signaling in liver, mice maintained on different diets were fasted 6 h before receiving an insulin injection. Anesthetized mice were opened, and a single piece of liver was excised and snap-frozen in liquid nitrogen as the untreated control. Within 4–5 min after injection via the portal vein with 10 U/kg human insulin (Eli Lilly, Indianapolis, IN, USA), another piece of liver was snap-frozen for subsequent protein extraction and western blot analysis.

**Construction of Adenoviral Vector encoding IP receptor**

To construct IP overexpressing adenoviral vector, mouse IP full-length cDNA was inserted into the pAd-Track-CMV construct, which was then sub-cloned into
pAd-Easy-1 adenoviral backbone vector through homologous recombination in BJ5183. To package the adenovirus, the adenoviral DNA was linearized by PacI restriction enzyme, and transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). After several rounds of propagation, recombinant adenovirus was purified by ultracentrifugation in cesium chloride gradient.

**Isolation of Liver Kupffer cells**

Liver Kupffer cells were prepared as previously described (20,21). The purity and viability of Kupffer cells were assessed by trypan blue and immunostaining.

**PG extraction**

PG extraction from liver tissue or culture medium was routinely performed in the laboratory (22).

**PCK1 activity**

PCK1 activity was examined as previously described (23).

**Generation of STZ-induced Diabetic Mice**

STZ-induced diabetic mice were produced using previously described methods (24).

**mRNA quantification by RT-PCR**

Total RNA from cultured hepatocytes or tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) then treated with RNase-free DNase (Takara, Dalian, China) at 37°C for 2 min to remove genomic DNA. The primers used for each target gene are summarized in Supplementary Table 1.
Statistical analysis

All statistical analysis was subjected to Student’s *t*-test. Time course studies were analyzed by two-way analysis of variance (ANOVA) followed by the Bonferroni post-test (GraphPad Prism 5 software). *P* < 0.05 was considered statistically significant. Data are represented as mean ± standard error of the mean (SEM).
Results

The COX-2/PGI2/IP axis is upregulated in liver in response to fasting and diabetic stress

We first examined the expression of all prostanoid receptors in metabolic tissues from chow-fed mice (Supplementary Fig. S1). All of the prostanoid receptors were variously expressed in liver except FP. Of note, TP and IP were abundantly detected in liver, white adipose tissue (WAT) and skeletal muscle. In contrast to the dominant constitutive expression of COX-1, only COX-2 was upregulated by fasting or administration of HFD to wild-type mice, and a similar induction was evident in Ob/Ob mice (Fig. 1A and B), with a corresponding induction of prostanoid formation (Fig. 1C). The most abundant product formed in the livers was PGI2 under physiological conditions. Further investigation of hepatocytes and Kupffer cell-enriched non-parenchymal cells (>80% by immunostaining), revealed that COX-2 expression was up-regulated in response to COX-1 deficiency (Fig. 1D and F), suggesting that products of COX-1 contribute to the expression of COX-2, which is consistent with previous observations (25). However, the deletion of either COX-1 or COX-2 reduced all prostanoids compared to WT controls (Fig. 1E and G). Interestingly, COX-2 expression and prostanoids production induced by high glucose concentrations appeared more robust in Kupffer cells than in hepatocytes (Fig. 1D-G). IP expression in liver was also elevated significantly in fasted, HFD fed and Ob/Ob
mice (Fig. 1H and I), however we did not observe any alterations of TP expression in metabolic organs in response to fasting and HFD challenge (data not shown).

**IP deficiency and high doses of ASA reduced fasting blood glucose levels in mice**

Given that the hepatic PGI$_2$/IP axis was activated in response to fasting and diabetic stresses, we first examined the effect of IP ablation on glucose metabolism in mice. IP deletion had no detectable effect on blood glucose levels in chow-fed mice, but significantly decreased blood glucose during fasting as measured at 4 h and 8 h (Fig. 2A). And hyperglycemic response to glucagon was markedly attenuated in IP deficient mice (Fig. 2B). Interestingly, mRNA and protein expression of key gluconeogenic genes in liver $G6Pase$ and $PCK1$, were consistently reduced in IP KO mice compared to WT littermates, before and after fasting (Fig. 2 C-E). PCK1 activity in liver was also impaired in IP KO mice in response to fasting (Fig. 2F). Moreover, we did not observe alterations of the key hepatic glycogenolytic gene for glycogen phosphorylase ($Pygfl$) in IP KO mice (Fig. 2G), suggesting that the fasting hypoglycemia in the mutants resulted from a defect of hepatic gluconeogenesis, not glycogenolysis. To further investigate whether IP disruption interferes with lipid metabolism and glycolysis in mice, we analyzed the blood lipid profiles and expression of enzymes involved in fatty acid synthesis, fatty acid oxidation (FAO) and glycolysis. No differences in blood cholesterol or triglycerides (TG) were detected between IP KO and WT in either fed or fasted states (Supplementary Fig. S2).
Additionally, the expression of key enzymes for *de novo* fatty acid synthesis, including ATP citratelyase (ACL), acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS) in livers, gastrocnemii and WAT, were unaltered in IP KO mice (Supplementary Fig. S3 A-C and Fig. S4 A-C). Moreover, IP deletion did not influence hepatic FAO, muscular glycolysis or glucose uptake by skeletal muscle or WAT in mice (Supplementary Fig. S5 A-C and Fig. S6 A-C). Taken together, these results suggest that IP is involved in hepatic gluconeogenesis in response to fasting.

High doses of ASA reportedly lower fasting blood glucose in type 2 diabetes mellitus (8,26). As expected, a marked reduction of PG formation in liver (including PGI$_2$, Supplementary Fig. S7 A) and a significant depression in fasting blood glucose were observed in Ob/Ob mice treated with 600 mg/L ASA in drinking water (Supplementary Fig. S7 B). Likewise, PCK1 and G6Pase expression in liver was also dramatically reduced by high doses of ASA, further suggesting that the hypoglycemic effect of ASA is due to suppression of hepatic gluconeogenesis (Supplementary Fig. S7 C). Interestingly, the decline of blood glucose in Ob/Ob mice by ASA was restored completely by administration of the IP agonist Cicaprost (Supplementary Fig. S7 D), suggesting that the hypoglycemic effect of ASA might be mediated, at least partially through inhibition of PGI$_2$/IP signaling.

To directly determine the effect of IP deficiency on gluconeogenesis *in vivo*, pyruvate tolerance tests (PTTs) were performed on age- and weight-matched male lean mice. IP
KO mice have a decreased gluconeogenic capacity (Fig. 3A). In addition, hypoglycemic response to insulin was more pronounced in IP KO mice compared with WT littermates (Fig. 3B and C). There was no marked improvements in glucose tolerance and normal glucose clearance in IP KO mice (Fig. 3D). However, we did not detect any overt differences in blood insulin and glucagon levels between IP KO and WT mice in either fed or fasted states, despite a sharp decrease in insulin and marked elevation of glucagon in response to fasting (Fig. 3E and F). No significant differences were detected in O2 consumption, CO2 and heat production, respiratory exchange ratio (RER) and activity between IP KOs and WTs (Supplementary Fig. S8 A-E).

**IP deficiency protected against both Streptozotocin (STZ)- and HFD-induced diabetes**

Multiple doses of STZ induced severe degeneration and necrosis of pancreatic β-cells (Fig. 4A) and islet shrinkage (Fig. 4B-C) in both IP KO and WT mice, as reflected by a striking reduction of insulin secretion (Fig. 4D). The elevation in blood glucose evoked by STZ in WT mice on regular chow was significantly blunted by IP deletion (Fig. 4E). No differences in insulin and glucagon levels were detected between IP KO and WT mice (Fig. 4D). Following HFD challenge, IP KO mice gained approximately the same body weight as their WT littermates (Fig. 4F) and exhibited normal body composition (Fig. 4G). There was no significant difference of blood glucose levels
between HFD challenged IP KO and WT mice (Fig. 4H). In response to both insulin and glucose tolerance tests following 16 weeks of HFD feeding, however, the plasma glucose levels were lower in IP KO mice compared with WT controls (Fig. 4I and J). Moreover, hepatic TG and CHO accumulation (Fig. 4K), and hepatocellular vacuolation (Fig. 4L) induced by HFD were also attenuated in IP KOs compared to littermate WT controls, while plasma levels of insulin and glucagon were unaltered (Fig. 4M). The increased expression of the pro-inflammatory genes, tumor necrosis factor-alpha (TNFα), monocyte chemotactic protein-1 (MCP-1) and interleukin 6 (IL-6), evoked in peritoneal macrophages and epididymal fat (eFat) by the HFD, was restrained by IP deletion (Fig. 4N and O). Thus, IP deletion attenuated the inflammatory response, and appeared to confer protection against STZ- and HFD-induced diabetes in mice, through improvement of glucose metabolism.

**Hepatic re-expression of the IP augmented gluconeogenesis and reduced insulin sensitivity in IP KO Mice**

We sought to determine whether re-expression/overexpression of the IP could rescue hepatic gluconeogenesis and insulin tolerance in IP KO mice. Adenoviruses encoding the IP were introduced into IP KO mice by tail vein injection. Adenoviral delivery of genes resulted in their hepatic expression, as indicated both by fluorescence intensity (Fig. 5A) and quantitative RT-PCR of IP expression (Fig. 5B). In the fasted state, adenovirus-mediated overexpression of IP (IP OV) in liver significantly improved
hypoglycemia (Fig. 5C) and elevated gluconeogenesis as reflected by pyruvate challenge in IP OVs (Fig. 5D). Thus, re-expression of the IP in liver alone substantially rescued the metabolic phenotype exhibited in mice globally deficient in the receptor (Fig. 5E).

**Disruption of the PKA/CREB pathway contributed to impaired gluconeogenesis consequent to IP Deficiency**

To explore the molecular mechanisms underlying IP-mediated regulation of gluconeogenesis in liver, we first determined whether the cAMP/PKA activity was regulated by PGI₂ in hepatocytes (27). In cultured primary hepatocytes, IP disruption dramatically reduced glucose production at different doses of insulin, resulting in suppression of glucose output (Fig. 6A). Hepatocyte intracellular cAMP was suppressed by IP deletion under basal and glucagon-evoked conditions (Fig. 6B). Similarly, glucagon-evoked phosphorylation of CREB, PCK1 and G6Pase (Fig. 6C and D) was also attenuated by IP deletion. In contrast, re-expression of the IP in livers of IP KOs (Fig. 6E) substantially rescued these phenotypes (Fig. 6F-I). The PKA inhibitor, H-89, lacked the augmentation of CREB phosphorylation induced by Ad-IP and partially inhibited the induction of PCK1 and G6Pase (Fig. 6G and H). In addition, re-expression of IP boosted hepatic glucose production (HGP, from 54 ± 3.9 µg·mg⁻¹·h⁻¹ to 87 ± 4.8 µg·mg⁻¹·h⁻¹), which could be depressed only 29.8% by H-89 treatment (from 87 ± 4.8 µg·mg⁻¹·h⁻¹ to 61 ± 3.2 µg·mg⁻¹·h⁻¹) in Ad-IP infected
hepatocytes. However, this remained higher than that in GFP-expressing hepatocytes (Fig. 6I), indicating that additional IP-mediated mechanism, independent of the cAMP/PKA pathway (Fig. 6J), may influence hepatic gluconeogenesis.

**IP deficiency increases AKT activation in liver**

In addition to glucagon/CREB signaling, hepatic glucose production and metabolism is also regulated by the insulin/AKT/FoxO1 axis. In the fed state, increased insulin secretion activates the AKT pathway in hepatocytes, which in turn, phosphorylates and inhibits FoxO1, which is subjected to ubiquitination and degradation in the cytoplasm, resulting in reduced expression of gluconeogenic genes, PCK1 and G6Pase (28). Under basal conditions, the level of phosphorylation of AKT (Ser473 and Thr308) is quite low in primary hepatocytes from WT mice. However, deletion of the IP in primary hepatocytes markedly increased phosphorylation of AKT, FoxO1 (Ser253) and GSK3β (Ser9, another AKT downstream substrate) even in the absence of insulin stimulation (Fig. 7A and B). Similar results were observed in livers obtained from mice fed a chow diet or HFD (Supplementary Fig. S9 A and B). Again, the expression levels of PCK1 and G6Pase decreased in IP KO hepatocytes (Fig. 7A). However, we did not detect alterations of IRS-1 protein expression and its tyrosine phosphorylation in IP deficient hepatocytes (Fig. 7A and B)-consistent with unchanged plasma insulin levels in the IP KO mice (Fig. 3E). Thus IP deletion appeared to modulate AKT activity independent of an effect on the insulin receptor.
The AKT inhibitor IV, which efficiently restrained phosphorylation of AKT at both Ser-473 and Thr-308 (Supplementary Fig. S10A and B), blocked the augmented phosphorylation of AKT and FoxO1 in IP deficient hepatocytes (Fig. 7C and D), but did not completely abolish the differences in expression of PCK1 and G6Pase between the two genotypes (Fig. 7C and D). Knockdown of AKT in primary hepatocytes further confirmed the findings described above (Supplementary Fig. S11).

In addition, IP disruption in cultured hepatocytes reduced HGP approximately by half (Fig. 7E). This corresponds to a reduction of approximately one fifth following treatment with AKT inhibitor IV (215 ± 10.83 µg·mg⁻¹·h⁻¹ vs. 175 ± 6.7 µg·mg⁻¹·h⁻¹), which was consistent with the premise that elevated activation of AKT contributed to suppression of hepatic gluconeogenesis in IP KO mice (Fig. 7F).

PGI₂ modulated hepatic gluconeogenesis via the PI3K/PKCζ/TRB3 pathway to depress AKT

The activity of AKT can be modulated through its interaction with various binding partners (29). For example, AKT activity can be down-regulated by carboxy-terminal modulator protein (CTMP) (30) and pseudokinase tribble3 (TRB3), an endogenous AKT inhibitor that binds to AKT and prevents insulin-mediated AKT phosphorylation (31). We observed that TRB3 expression at both mRNA (Supplementary Fig. S12 A and B) and protein (Fig. 8A and Supplementary Fig. S13A) levels was markedly suppressed in livers from IP KO mice under both fed and fasted conditions, and this
expression could be rescued by hepatic re-expression of the IP in the KOs (Fig. 8B and Supplementary Fig. S13 B). Deletion or re-expression of IP in hepatocytes failed to influence expression of PDK1 and CTMP (data not shown). Insulin also induces the expression of TRB3, which, in turn, modulates AKT activity to maintain normal glucose metabolism (31). Insulin treatment led to rapid AKT phosphorylation (peaking at 2 h) followed by subsequent de-activation, while TRB3 induction lagged behind AKT phosphorylation (Fig. 8C and Supplementary Fig. S13 C-D), which was consistent with the notion that TRB3 negatively regulates AKT activation (32). In addition to augmenting activation of AKT, the dynamic expression of TRB3 was reduced in IP deficient hepatocytes (Fig. 8C).

TRB3 expression is dependent on the activity of PI3 kinase (PI3K) (33) and its downstream atypical PKC, PKCζ (32). As with other GPCRs, stimulation of the IP can activate Class 1B PI3K, (PI3Kγ, catalytic unit p110γ), through heterotrimeric G proteins Gα and Gβγ that bind to the pleckstrin homology domain in the NH2-terminal region of PI3Kγ (34). Phosphorylation of PKCζ was decreased in IP deficient hepatocytes (Fig. 8D and Supplementary Fig. S13E). Re-expression of the IP significantly elevated phosphorylation of PKCζ (Fig. 8E and Supplementary Fig. S13F), while IP specific antagonist CAY-10441, PI3K inhibitor LY294002 and PKC inhibitor RO32-0432 all suppressed the induction of PKCζ phosphorylation (Fig. 8F). Similarly, differences in hepatic TRB3 expression resulting from deficiency or
re-expression of IP were blunted by PI3K inhibitor LY294002 and PKCζ inhibitor RO32-0432 (Supplementary Fig. S14 A and B), indicating that regulation of TRB3 by IP is mediated by the PI3K/PKCζ pathway. Knockdown of PKCζ in primary hepatocytes further confirmed our findings described above (Supplementary Fig. S15). Silencing of TRB3 (Ad-shTRB3) suppressed IP mediated-HGP by ~40% (from 99 ± 4.1 µg·mg⁻¹·h⁻¹ to 56 ± 4.9 µg·mg⁻¹·h⁻¹), while the combination of Ad-shTRB3 and the PKA inhibitor H-89 completely abolished HGP induced by IP re-expression in hepatocytes (Fig. 8G). Likewise, we also observed the suppression of PI3Kγ signaling and decreased phosphorylation of CREB in mice treated with high doses of ASA, and these trends were reversed by activation of IP (Supplementary Fig. S16). These findings implicated PI3Kγ/PKCζ/TRB3/AKT signaling in IP modulation of hepatic gluconeogenesis (Fig. 8H).

**Discussion**

In this study, we found that hepatic expression of COX-2, PGI₂ production and IP expression were enhanced under conditions associated with augmented hepatic gluconeogenesis (e.g., during fasting in WT mice, and in mice predisposed to diabetes due to either genetic mutations or pharmacological treatment). Disruption in this pathway, specifically by deleting the IP, inhibited hepatic gluconeogenesis and ameliorated diabetes for the latter conditions, while this phenotype could be rescued
in large part by hepatic re-expression of the IP. These findings establish a novel connection between PGI₂, a cardioprotective (35) and pro-inflammatory eicosanoid (27), and carbohydrate metabolism, which may explain, at least in part, the reported metabolic effects of NSAIDs in humans (8).

Inhibition of COX-2, the dominant source of PGI₂ biosynthesis in humans (36), has been reported to increase insulin sensitivity in both healthy(6) and overweight individuals (7). Pharmacological inhibition and genetic ablation of COX-2 in mice reduces fasting glucose and protects against STZ-induced diabetes (37). Treatment with high doses of ASA, which would be expected to inhibit both COX-1 and COX-2, ameliorates insulin resistance in patients with type 2 diabetes by reducing gluconeogenesis and stimulating peripheral glucose uptake (8), likely by inhibiting IKKβ/NFκB activity (26), which influences COX-2 transcription and activity. These observations are consistent with the notion that COX-2 dependent products play a functionally important role in the regulation of gluconeogenesis in humans.

We also found dysregulated expression of COX-2, PGI₂ biosynthesis and IP expression in the livers of fasted, diet-induced obese, and Ob/Ob mice. IP deficiency depressed fasting-induced hepatic gluconeogenesis and enhanced the hypoglycemic effect of insulin in mice, probably due to impaired counter regulatory response (38). Moreover, deletion of IP in mice slowed the progression of diabetes induced by either STZ or a high fat diet. Conversely, hepatic overexpression of IP in liver increased
gluconeogenesis, resulting in insulin resistance. IP deletion in primary hepatocytes resulted in the diminished glucagon-mediated phosphorylation of CREB and diminished transcription of gluconeogenic genes, and this phenotype was rescued by re-expression of the IP in liver. These results suggest that COX-2/PGI2/IP axis influences gluconeogenesis, at least in part, by enhancing the glucagon-mediated pathway. Conversely, insulin has been shown to depress hepatic gluconeogenesis through the IR/IRS-mediated PI3K/AKT/FoxO1 pathway (39). Indeed, IP ablation also increased AKT/FoxO1 signaling in response to insulin in cultured hepatocytes, and in livers from starved and HFD-challenged mice. IP activation also perturbs hepatic insulin signaling to regulate gluconeogenesis through modulation of AKT activity. Class 1A PI3Ks (including PI3Kα and PI3Kβ) are activated by receptor tyrosine kinase (RTK, such as insulin receptor), whereas Class 1B PI3K (i.e., PI3Kγ) is activated by the binding of p110γ to the Gβγ unit of GPCR(34). Meanwhile, p110γ interacts physically with PKCζ (40). We did observe that the elevation of TRB3 expression induced by activation of IP (a Gαs coupled receptor) could be abrogated by both PI3K and PKCζ inhibitors, consistent with previous studies, indicating that hepatic TRB3 could be regulated by PI3K and PKCζ (32,41). Moreover, we did not observe any effects of cAMP/CREB on TRB3 expression by CHIP assay (Supplementary Fig. S17), which is consistent with that H89 treatment was unable to entirely block HGP induced by IP re-expression. Collectively, these results indicated
that activation of the IP regulated hepatic gluconeogenesis via both cAMP/PKA/CREB and PI3Kγ/PKCζ/TRB3/AKT pathways, and a combination of PKA inhibitor (H89) treatment and TRB3 silencing could entirely restrain IP activation-induced HGP.

PGI₂ displays both pro-inflammatory (e.g. rheumatoid arthritis) and anti-inflammatory properties (e.g. atherosclerosis) properties, depending upon the inflamed organs and the pathological models (27). Our results indicated that PGI₂/IP may modulate the pathological process of excessive hepatic gluconeogenesis in diabetes. As one of the effective drugs for management of pulmonary arterial hypertension, synthetic PGI₂-Epoprostenol has been reported to elevate serum glucose in humans and animals (42), while treatment with another PGI₂ analogue, Iloprost, significantly reduced lactate, and slightly increased glucose, in patients with critical limb ischemia (43), thereby indirectly implicating IP signaling in gluconeogenesis. However, PGI₂ in fat tissue also promotes adipose cell differentiation (44) and de novo BAT recruitment in WAT (45) through another natural nuclear receptor, peroxisome proliferator-activated receptor γ (PPARγ). Interestingly, treatment with beraprost, a PGI₂ analogue, ameliorated diabetic complications such as nephropathy in severely diabetic rodents by reduction of inflammation in peripheral tissues through upregulation of PPARγ (46) and perhaps PPARδ (47). Subsequent activation of PPARγ, which reduces a flux of free fatty acid and cytokines from adipose tissue to
the liver, results in increased insulin sensitivity in diabetes (48). However, a slight increase of hepatic PPARγ expression was observed in IP KO mice (data not shown), suggesting that beraprost might regulate PPARγ directly (49), rather than through IP.

NSAIDs, particularly those specific for inhibition of COX-2, can influence carbohydrate metabolism, specifically gluconeogenesis, in humans. Amongst the prostanoids, COX-2 is the dominant contributor to biosynthesis of PGI2, and PGI2 is the major product of mouse hepatocytes under physiological conditions. Here, we show that the IP for PGI2 modulates hepatic gluconeogenesis through both the Gαs/PKA/CREB and Gβγ/PI3Kζ/PKCζ/TRB3/AKT pathways (Fig. 8H) and that disruption of this receptor confers protection against the progression of diabetes by inhibition of hepatic gluconeogenesis. These observations provide a mechanistic rationale for clinical observations suggesting a role for COX-2 inhibition in the regulation of carbohydrate metabolism.
Acknowledgements

This work was supported by grants from the Ministry of Science and Technology of China (2012CB945100, 2011CB503906, 2011ZX09307-302-01 and 2012BAK01B00); the National Natural Science Foundation of China (81030004); NSFC-CIHR joint grant (NSFC81161120538 and CIHR-CC117951); the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-EW-R-09); and the Clinical Research Center at the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences (CRC2010007). Y.Y. was supported by the One Hundred Talents Program of the Chinese Academy of Sciences (2010OHTP10) and Pujiang Talents Program of Shanghai Municipality (11PJ1411100).

The authors declare that they have no conflict of interest.

S.Y, Q.Z and Y.Y. designed the research associated with the project. S.Y., Q.Z. X. Z., J. T., Y.W., Y. Z. and J. Z. performed experiments. J.Y., F.G., Y. L. and G.A.F. provided important reagents. S.Y., G.A.F. and Y.Y. wrote the manuscript. Y.Y. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Dr. Shengzhong Duan (Institute for Nutritional Sciences Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) for technical assistance.
References


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Figure legends

**FIG. 1. Alterations of the COX-2/PGI2/IP axis in liver response to fasting and diabetic stress.** A: Western blot analysis of COX-1 and COX-2 in liver tissue from regular chow diet fed (CHOW), high-fat diet (HFD) treated and Ob/Ob mice, under fed or 12h fasting conditions. B: Densitometric analysis of the abundance of COX-1 and COX-2 as in (A).*P < 0.05; **P < 0.01 vs. WT, n = 4. C: PG profile of liver tissue from normal fed (Fed), fasting, high-fat diet (HFD) treated and Ob/Ob mice (12 week-old). *P < 0.05 vs. Fed group, n = 8. D: Expression of COX-1 and COX-2 in primary hepatocytes from WT, COX-1 KO and COX-2 KO mice. E: PG profile of primary hepatocytes at basal and high glucose-stimulated conditions. *P < 0.05 vs. WT; #P < 0.05 vs. basal condition (5 mM glucose), n=6. F: Expression of COX-1 and COX-2 in Kupffer cells from WT, COX-1 KO and COX-2 KO mice. G: PG profile of Kupffer cells at basal and high glucose-stimulated conditions. *P < 0.05 vs. WT; #P < 0.05 vs. basal condition (5 mM glucose), n = 6. H: mRNA expression of IP in liver from regular chow diet fed (Fed), fasting, HFD treated and Ob/Ob mice.*P < 0.05 vs. Fed group, n = 8. I: Schematic diagram for changes of COX-2/PGI2/IP axis in livers under fasting or diabetic stresses.

**Fig. 2. Impaired hepatic gluconeogenesis in IP KO mice.**

A: Blood glucose changes in response to fasting in IP KO and WT littermates. * P< 0.05 vs. WT, n = 8. B: Blood glucose changes in response to glucagon challenge in IP KO and WT littermates. *P < 0.05 vs. WT, n = 6–8. C: mRNA expression levels of hepatic PCK1 and G6Pase in IP KO and WT littermates after 8 h of fasting; *P < 0.05 vs. WT, n = 6. D: Immunoblot of PCK1 and G6Pase expression in livers from IP KO
and WT littermates during fasting. E: Densitometric analysis for the abundance of PCK1 and G6Pase as in (D). \*P < 0.05; \**P < 0.01 vs. WT, n = 4. F: PCK1 enzyme activity in livers from IP KO and WT littermates after fasting 8 h. \**P < 0.01 vs. WT, n = 6. G: mRNA expression of glycogen phosphorylase (Pygl) in livers from IP KO and WT littermates after 8h fasting. \#P < 0.05 vs Fed controls.

**Fig. 3. Enhanced hypoglycemic response to insulin in IP deficient mice.**

A: Blood glucose changes in response to pyruvate challenge in IP KO and WT littermates. \*P < 0.05; \**P < 0.01 vs. WT, n = 6–8. B: Percentage of blood glucose changes from baseline to insulin challenge in IP KO and WT littermates. \*P < 0.05 vs. WT, n = 8–10. C: Area under curve (AUC) for (A). \*P < 0.05 vs. WT, n = 8–10. D: Blood glucose changes during GTT in IP KO and WT littermates. \*P < 0.05 vs. WT, n = 8–10. E: Plasma insulin and F: Plasma glucagon levels in WT and IP KO mice receiving regular chow diet or fasting for 12h. \#P < 0.05 vs. chow-fed, n = 8.

**Fig. 4. IP Deficient Mice are resistant to STZ and HFD-induced diabetes.**

A: Representative hematoxylin and eosin staining of pancreatic sections from STZ-treated IP KO and WT mice. Scale, 100 µm. B: Representative immunostaining of insulin in pancreatic sections from STZ-treated IP KO and WT mice. Scale bar, 100 µm. C: Quantification of insulin staining in (B). \#P < 0.05 vs. Control, n = 6. D: Plasma insulin and glucagon levels of STZ-treated IP KO and WT mice. \#P < 0.05 vs. Control, n = 8. E: Blood glucose changes of IP KO and WT mice after STZ treatments. \*P < 0.05; \**P < 0.01 vs. WT, n = 8. F: Body weight of IP KO and WT mice challenged with HFD. Mice were weighed every week following HFD treatment, n = 8. G: Body composition of IP KO and WT mice. Mice before (CHOW) and after 16
weeks HFD treatment were subjected to fat/lean evaluation by dual x-ray absorptiometry (DEXA), n = 8. H: Blood glucose concentration of IP KO and WT mice fed with HFD. n=8-10 I: Insulin tolerance test on IP KO and WT control mice fed with HFD for 16 weeks. *P < 0.05 vs. WT, n= 8. J: Glucose tolerance test on IP KO and WT mice fed with HFD for 16 weeks. **P < 0.01 vs. WT control, n = 8. K: Hepatic triglyceride (TG) and total cholesterol (CHO) contents of IP KO and WT controls fed with either regular chow diet (CHOW) or HFD for 16 weeks. *P < 0.05; **P < 0.01 vs. WT control; #P < 0.05 vs. CHOW, n = 8. L: Representative HE stainings of livers from HFD-treated IP KO and WT mice. M: Plasma insulin and plasma glucagon levels in WT and IP KO mice after HFD for 16 weeks. #P < 0.05 vs. chow-fed, n = 8. N: mRNA levels of TNF\(_{\alpha}\), MCP-1 and IL-6 in peritoneal macrophages from IP KO and WT mice before (CHOW) and after 16 weeks HFD treatment. *P < 0.05, **P < 0.01 vs. WT control; #P < 0.05 vs. CHOW, n = 8. H: mRNA levels of TNF\(_{\alpha}\), MCP-1 and IL-6 in WAT from IP KO and WT mice before (CHOW) and after 16 weeks HFD treatment. *P < 0.05, **P < 0.01 vs. WT control; #P < 0.05 vs. CHOW, n = 8.

**Fig. 5.** Hepatic re-expression of IP in liver restored fasting-induced hypoglycemia and reduced insulin sensitivity and glucose tolerance in mice. A: Representative imaging of IP KO mice expressing adenovirally encoded IP (IP OV) and GFP (GFP). Adenovirus was infused by tail vein and images were analyzed on day 7. B: IP expression in livers from IP KO mice after 7 days of adenovirus infusion. *P < 0.05, n = 6. C: Effect of IP adenovirus (IP OV) infusion on plasma glucose
levels in IP KO mice in response to fasting. *P < 0.05 vs. GFP controls, n = 6. D: Pyruvate tolerance test of IP KO mice expressing adenovirus encoded with GFP or IP (IP OV). *P < 0.05; **P < 0.01 vs. GFP, n = 8. E: ITT of IP KO mice expressing adenovirus- encoded GFP or IP (IP OV). *P < 0.05; **P < 0.01 vs. GFP, n = 8.

**Fig. 6.** IP ablation attenuated PKA-mediated CREB phosphorylation and impaired hepatic glucose production (HGP).

A: Glucose production in primary hepatocytes isolated from WT and IP KO mice. Cells were incubated for 6 h with glucose production buffer supplemented with insulin. *P < 0.05 vs. WT, n = 6. B: Intracellular cAMP concentrations in primary hepatocytes exposed to glucagon (100 nM) prepared from WT and IP KO mice. *P < 0.05 and **P < 0.01 vs. WT. #P < 0.05 vs. Control group, n = 6. C: Effect of IP deletion on phosphorylation of CREB (p-CREB) and expression of PCK1 and G6Pase in primary hepatocytes isolated from WT and IP KO mice. Experiments were repeated 3 times. D: Densitometric quantitation of hepatic expression of p-CREB, PCK1 and G6Pase presented in (C). *P < 0.05 vs. Control; #P < 0.05 vs. WT, n = 4. E: mRNA expression levels of IP in GFP or IP expressing (OV) adenovirus-infected IP KO hepatocytes, **P < 0.01, n = 3. F: Effect of IP re-expression (IP OV) on intracellular cAMP level in hepatocytes in response to glucagon (100 nM) challenge. **P < 0.01 vs. GFP vector; #P < 0.05 vs. Control, n = 6. G: Western blot analysis of p-CREB, PCK1 and G6Pase in primary hepatocytes infected with adenovirus encoded GFP or IP (IP OV) with or without pretreatment with the PKA inhibitor H-89. H: Densitometric quantitation of hepatic expression of p-CREB, PCK1 and G6Pase as
presented in (G). *P < 0.05 vs. Control; #P < 0.05 vs. GFP, n = 4. I: Effect of H-89 on glucose production from IP re-expressed hepatocytes, **P < 0.01 vs. GFP vector, #P < 0.05 vs. PBS Control, n = 6. J: Schematic diagram of IP-mediated hepatic gluconeogenesis through the cAMP/PKA/CREB pathway.

**Fig. 7.** AKT/FoxO1-mediated suppression of hepatic gluconeogenesis was enhanced in IP KO mice.

A: Western blot analysis of hepatic insulin signaling in primary hepatocytes isolated from IP KO and WT mice. B: Densitometric quantitation of hepatic phosphorylation of IRS1, AKT, FoxO1 and GSK3β compared against total expression, and hepatic expression of PCK1 and G-6-P by normalized to β-actin as seen in (A). *P < 0.05, **P < 0.01 vs. PBS control; #P < 0.05 vs. WT, n = 4. C: Effect of AKT inhibitor on phosphorylation of AKT/FoxO1 and expression of PCK1 and G6Pase in hepatocytes isolated from IP KO and WT mice. D: Densitometric quantitation of hepatic phosphorylation of AKT, and FoxO1 compared against total expression, and hepatic expression of PCK1 and G6Pase normalized to β-actin as seen in (C). *P < 0.05, **P < 0.01 vs. PBS control; #P < 0.05 vs. WT, n = 4. E: Effect of AKT inhibitor IV on glucose production from WT and IP KO hepatocytes. *P < 0.05 vs. WT, #P < 0.05 vs. vehicle Control, n=6. F: Schematic diagram of IP-mediated hepatic gluconeogenesis through the PI3Kγ/AKT/FoxO1 pathway.

**Fig. 8.** IP/PI3Kγ/PKCζ/TRB3 signaling axis was involved in hepatic gluconeogenesis. A: Western blot analysis of TRB3 in WT and IP KO hepatocytes cultured in the presence (Control) and absence (Starve) of serum. B: Effect of
re-expression of IP receptor on TRB3 expression in hepatocytes. C: Association of TRB3 expression with AKT phosphorylation in primary hepatocytes in response to insulin treatment. D: Effect of IP deletion on phosphorylation of PKCζ in primary hepatocytes from WT and IP KO mice. E: Effect of IP over-expression on phosphorylation of PKCζ in hepatocytes infected with Ad-GFP (GFP) or Ad-IP (IP OV). F: Effect of CAY-10441 (IP antagonist) on phosphorylation of PKCζ. G: Effect of both TRB3 shRNA and H-89 treatment on HGP of IP re-expressed hepatocytes (IP OV). Inset shows mRNA expression levels of TRB3 in hepatocytes infected with shTRB3 adenovirus. **P < 0.01 vs. vehicle control, #P < 0.05 vs. GFP control, n=6. H: Schematic diagram of IP-mediated hepatic gluconeogenesis through both PI3Kγ/PKCζ/TRB3/AKT and cAMP/CREB pathways.
Fig. 1

A

WT
Fed Fasting Fed Fasting Fed Fasting

Ob/Ob
COX-1
COX-2
α-tubulin

CHOW HFD CHOW

B

Relative protein level

Fed Fasting

COX-1
COX-2

CHOW HFD CHOW HFD CHOW

C

pg/μg protein

Fed Fasting HFD Ob/Ob

COX-1
COX-2
β-actin

Glucose 5mM 25mM

D

WT COX-1 KO COX-2 KO

WT COX-1 KO COX-2 KO

WT COX-1 KO COX-2 KO

E

pg/μg protein

6-keto-PGF1α 1α TXB2 PGD2 PGE2

6-keto-PGF1α 1α TXB2 PGD2 PGE2

6-keto-PGF1α 1α TXB2 PGD2 PGE2

F

COX-1
COX-2
β-actin

Glucose 5mM 25mM

G

pg/μg protein

6-keto-PGF1α 1α TXB2 PGD2 PGE2

6-keto-PGF1α 1α TXB2 PGD2 PGE2

6-keto-PGF1α 1α TXB2 PGD2 PGE2

H

Relative mRNA level

Fed Fasting HFD Ob/Ob

I

Hepatocytes

254x338mm (300 x 300 DPI)
Fig 4

A  Control   STZ
   WT   IP KO
   H&E

B  Control   STZ
   Insulin(IHC)

C  WT
   IP KO
   STZ
   Area (arbitrary units)

D  Insulin
   WT   IP KO
   pg/mL

E  Glucagon
   WT   IP KO
   pg/mL

F  WT   IP KO
   Blood glucose (mg/dL)
   Time (Day)

G  Fat Mass
   Lean Mass
   WT   KO   CHOW   HFD

H  Blood glucose (mg/dL)
   WT   IP KO
   Time (Day)

I  Blood glucose (mg/dL)
   WT   IP KO
   Time (min)

J  WT   IP KO
   Blood Glucose (mg/dL)
   Time (Day)

K  WT   IP KO
   Insulin
   pg/mL

L  Glucagon
   WT   IP KO
   pg/mL

M  CHOW   HFD
   TNF-α

N  CHOW   HFD
   IL-6

O  CHOW   HFD
   MCP1

P  CHOW   HFD
   TNF-α

Q  CHOW   HFD
   IL-6

R  CHOW   HFD
   MCP-1

254x338mm (300 x 300 DPI)
Fig 5

A

B

C

D

E
Fig 7

A

Ins | WT | IP KO
---|---|---
P-IRS1 |  |  |
IRS1 |  |  |
P-AKT(473) |  |  |
P-AKT(308) |  |  |
AKT |  |  |
P-FoxO1 |  |  |
FoxO1 |  |  |
P-GSK3β |  |  |
GSK3β |  |  |
PCK1 |  |  |
G6Pase |  |  |
β-actin |  |  |

B

Control | Ins
---|---
WT | IP KO | WT | IP KO | WT | IP KO | WT | IP KO | WT | IP KO | WT | IP KO

C

AKT inhibitor IV | WT | IP KO
---|---|---
P-AKT(473) |  |  |
P-AKT(308) |  |  |
AKT |  |  |
P-FoxO1 |  |  |
FoxO1 |  |  |
PCK1 |  |  |
G6Pase |  |  |

D

Control | AKT inhibitor IV
---|---
WT | IP KO | WT | IP KO | WT | IP KO | WT | IP KO | WT | IP KO | WT | IP KO | WT | IP KO

E

HGP (µU/g/h)

Control AKT inhibitor IV

F

Insulin receptor

Cytoplasm

PI3K

P-AKT

FoxO1

PCK1

G6Pase

Nucleus

254x338mm (300 x 300 DPI)
Fig 8

A

TRB3
β-actin
WT
IP KO

B

TRB3
β-actin
GFP
IP OV

C

Ins
WT
IP KO
0 1 2 4 6 8 0 1 2 4 6 8
P-AKT(473)
AKT
TRB3
β-actin

D

WT
IP KO
P-PKCζ
PKCζ

E

GFP
IP OV
GFP
IP OV
P-PKCζ
PKCζ
Con
CAY

F

DMSO
LY
RO
GFP
IP OV
GFP
IP OV
GFP
IP OV
GFP
IP OV
P-PKCζ
PKCζ
Con
INS

G

H

Insulin
IRS
P3K
p-Akt
PKCζ
p-FoxO1
FoxO1
P-CREB
PKA
GAPDase
**Supplementary Table 1 Primer for RT-PCR**

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**Supplementary Table 2** Primer for Real Time-PCR

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Supplementary Figure 1 Expression of PG receptors in metabolic organs of mice. RT-PCR analysis of mRNA level in mouse liver, white adipose tissue (WAT) and gastrocnemius from mice.
Supplementary Figure 2 Plasma lipid profile of IP KO mice and WT littermates. Plasma was collected from 8 week-old mice that were fed with chow diet or underwent overnight fasting: CHO, HDLC, LDLC and TG were measured routinely, n = 8.
Supplementary Figure 3 Effect of IP deficiency on de novo fatty acid synthesis in mice during fasting. mRNA levels of lipogenic enzymes FAS, ACC1 and ACL in liver (A), WAT (B) and gastrocnemius muscle (C) from both IP KO and WT mice were measured after 12 h fasting. n = 8.
Supplementary Figure 4  Effect of IP deficiency on protein expression of FAS and ACC1 in metabolic organs in mice. Protein levels of lipogenic enzymes FAS, ACC1 in liver (A), WAT (B) and gastrocnemius muscle (C) from both IP KO and WT mice were measured after 12 h fasting.
Supplementary Figure 5  Effects of IP deficiency on expression of hepatic fatty acid oxidation, muscle glycolysis and GLUT4 expression in liver and muscle. (A) The hepatic expression of CPTL1, MCAD, and PPARα in IP KO and WT mice after 12 h fasting. (B) The mRNA levels of Pkfm, HK2, and PK in the gastrocnemius from IP KO and WT mice during feeding (Fed), and after 12 h fasting. (C) The mRNA levels of GLUT4 in the gastrocnemius and WAT from IP KO and WT during feeding (Fed) and after 12 h fasting, n = 8.
Supplementary Figure 6 Effects of IP deficiency on protein expression of hepatic PPARα, HK2 in muscle and GLUT4 in muscle and WAT. (A) The hepatic expression of PPARα in IP KO and WT mice after 12 h fasting. (B) The protein levels of HK2 in the gastrocnemius from IP KO and WT mice during feeding (Fed), and after 12 h fasting. (E) The protein levels of GLUT4 in the gastrocnemius and WAT from IP KO and WT during feeding (Fed) and after 12 h fasting.
Supplementary Figure 7  High doses of ASA reduces blood glucose in Ob/Ob mice by suppression of hepatic PG12 production.

(A) Effect of high-dose ASA (600mg/L in drinking water) on hepatic PG profile in Ob/Ob mice. *P < 0.05 vs. Control; n = 6. (B) Effect of high-dose ASA on fasting blood glucose levels in Ob/Ob mice. *P < 0.05 vs. Control; n = 6. Wk, week. (C) Effect of high-dose ASA on protein (Left panel) and mRNA expression (Right panel) of hepatic PCK1 and G6Pase in Ob/Ob mice. *P < 0.05 vs. Control. (D) Effect of Cicaprost (Cica) on fasting blood glucose levels in ASA-treated Ob/Ob mice. *P < 0.05 vs. ASA treated Ob/Ob mice; n = 6.
Supplementary Figure 8  Fuel homeostasis of IP KO and WT littermates.
Oxygen consumption (VO₂) (A), Carbon dioxide production (VCO₂) (B), Activity (C), Heat production (D) and Respiratory exchange ratio (RER, E) of IP KO and WT mice were recorded in metabolic cages during regular light/dark cycles. n = 8.
Supplementary Figure 9  Effects of IP deletion on hepatic expression of P-AKT and P-FoxO1 in regular diet and HFD-fed mice. (A) Western blot analysis of hepatic P-AKT and P-FoxO1 in chow-fed IP KO and WT mice. (B) Western blot analysis of hepatic P-AKT and P-FoxO1 in HFD-challenged IP KO and WT mice.
Supplementary Figure 10  Effects of AKT inhibitor IV on phosphorylation of AKT in primary hepatocytes. (A) Western blot analysis of phosphorylation of AKT inhibitor IV-treated hepatocytes isolated from IP KO mice infected with either GFP or IP OV adenovirus for 24 h. (B). Densitometric quantitation of phosphorylation of AKT as shown in (A). *P < 0.05 vs. Control; †P < 0.05 vs. GFP, n = 6.
Supplementary Figure 11  Effects of knockdown of AKT on phosphorylation of AKT in primary hepatocytes. Western blot analysis of phosphorylation of AKT, FoxO1, PCK1 and G6Pase in primary hepatocytes treated with indicated SiRNA for 48h, Scram,Scram SiRNA; SiAKT, AKT SiRNA.
Supplementary Figure 12 Effects of IP deletion on hepatic expression of TRB3 in mice. mRNA expression levels of TRB3 in livers from chow-fed (A) and 12h fasting (B) WT and IP KO mice, n = 6.
Supplementary Figure 13 Deletion of IP modulates TRB3 expression via the PI3K/PKCζ pathway. (A) Densitometric quantitation of hepatic expression of TRB3 as presented in Fig. 8A. *P < 0.05 vs. WT. n = 6. (B) Densitometric quantitation of hepatic expression of TRB3 as presented in Fig. 8B. *P < 0.05 vs. GFP. n = 3. (C, D) Densitometric quantitation of expression of P-AKT(473) and TRB3 as presented in Fig. 8C. *P < 0.05 vs. WT. n = 6. (E) Densitometric quantitation of expression of P-PKCζ as presented in Fig. 8D. *P < 0.05 vs. WT. n = 6. (F) Densitometric quantitation of expression of P-PKCζ as presented in Fig. 8E. *P < 0.05 vs. WT. n = 6.
Supplementary Figure 14 Effect of IP deletion on expression of TRB3 in primary hepatocytes treated with LY294002 or RO32-0432. (A) Effect of LY294002 or RO32-0432 on TRB3 expression in primary hepatocytes from IP KO and WT mice, cells were pre-treated with 100nM insulin for 30min. *$P < 0.05$ vs. WT, n = 6. (B) Effect of LY294002 or RO32-0432 on TRB3 expression in Ad-IP (IP OV) or Ad-GFP (GFP) infected hepatocytes, cells were pre-treated with 100nM insulin for 30min. *$P < 0.05$ vs. GFP, n = 6.
Supplementary Figure 15  Effects of knockdown of PKCζ on phosphorylation of TRB3 in primary hepatocytes. Western blot analysis of phosphorylation of PKCζ, and RTB3 in primary hepatocytes treated with indicated SiRNA for 48h, Scram, Scram SiRNA; SiPKCζ, PKCζ SiRNA.
Supplementary Figure 16  Effect of Cicaprost on phosphorylation of PKCζ, AKT and CREB in livers from high-doses ASA treated Ob/Ob mice. Western blot analysis of phosphorylation of PKCζ, AKT(473) and CREB in livers from high-doses ASA treated Ob/Ob mice injected with Cicaprost (Cica, 4µg/25g/day) or DMSO for 7 days.
Supplementary Figure 17  No CREB binding in 5′ UTR of TRB3 gene. (A) 3 potential CREB binding sites (Site 1, 2, 3) in 5′ UTR of the TRB3 gene predicted at CREB Target Gene Database (http://natural.salk.edu/CREB/). (B) Evaluation of 3 potential CREB binding sites in 5′ UTR of TRB3 gene by ChIP assay. DNA-protein complex was immunoprecipitated by antibodies against p-CREB or immunoglobulin G (IgG (control), and specific real-time PCRs were performed to amplify targeting binding bands. P=NS, n = 6.