Adenine nucleotide-mediated regulation of hepatic PTP1B activity in mouse models of type 2 diabetes

Xiao Yang¹ · Yang Zhao¹ · Qi Sun¹ · Yunxia Yang¹ · Yan Gao¹ · Wenhao Ge¹ · Junhao Liu¹ · Xi Xu¹ · Dan Weng¹ · Shiming Wang¹ · Jianfa Zhang¹

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Abstract

Aims/hypothesis Plasma 5'-AMP (pAMP) is elevated in mouse models of type 2 diabetes. However, the metabolic regulatory role of adenine nucleotides in type 2 diabetes remains unclear.

Methods Adenine nucleotides and their metabolites in plasma and liver were examined by HPLC. ¹H NMR-based metabolomics analysis was performed to explore the changes of metabolites in mouse models of type 2 diabetes. Na⁺/K⁺ ATPase and Na⁺/H⁺ exchanger activity were measured in response to adenine nucleotide metabolites. Human recombinant protein tyrosine phosphatase 1B (PTP1B) was used for enzyme kinetic assays. Protein binding assays were performed with microscale thermophoresis. The intracellular pH of hepatocyte AML12 cell lines was measured using the BCECF-AM method. We also analysed pAMP levels in participants with type 2 diabetes.

Results Elevation of pAMP was a universal phenomenon in all mouse models of type 2 diabetes including *db/db* vs lean mice ($13.9 \pm 2.3 \mu\text{mol/l}$ vs $3.7 \pm 0.9 \mu\text{mol/l}$; $p < 0.01$), *ob/ob* vs lean mice ($9.1 \pm 2.0 \mu\text{mol/l}$ vs $3.9 \pm 1.2 \mu\text{mol/l}$; $p < 0.01$) and high-fat diet/streptozotocin-induced vs wild-type mice ($6.6 \pm 1.5 \mu\text{mol/l}$ vs $4.1 \pm 0.9 \mu\text{mol/l}$; $p < 0.05$); this elevation was required for the occurrence of hyperglycaemia in obese mice. ¹H NMR-based metabolomics study following HPLC analysis revealed that the metabolite profile in wild-type mice treated with 5'-AMP was similar to that in *db/db* diabetic mice, especially the accumulation of a large quantity of ATP and its metabolites. The glucose-lowering drug metformin reduced the severity of hyperglycaemia both in 5'-AMP-induced wild-type mice and *db/db* mice. Metformin decreased the accumulation of liver ATP but not its metabolites in these hyperglycaemic mice. ATP and metformin reciprocally change cellular pH homeostasis in liver, causing opposite shifts in liver activity of PTP1B, a key negative regulator of insulin signalling. Furthermore, pAMP levels were also elevated in individuals with type 2 diabetes ($45.2 \pm 22.7 \text{ nmol/l}$ vs $3.1 \pm 1.9 \text{ nmol/l}$; $p < 0.01$).

Conclusions/interpretation These results reveal an emerging role for adenine nucleotide in the regulation of hyperglycaemia and provide a potential therapeutic target in obesity and type 2 diabetes.

Keywords ATP · Hyperglycaemia · Metformin · Obesity · pH_i · PTP1B

Abbreviations

AMPK AMP-activated protein kinase

Xiao Yang, Yang Zhao and Qi Sun contributed equally to this work.

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✉ Jianfa Zhang
jfzhang@mail.njust.edu.cn

¹ Center for Molecular Metabolism, Nanjing University of Science & Technology, 200 Xiaolingwei, Nanjing 210094, China

ANM	Adenine nucleotide metabolite
BCECF-AM	(2',7'-Bis-[carboxyethyl]-5-[and-6]-carboxyfluorescein)-tetraacetoxymethyl ester
G6Pase	Glucose-6-phosphatase
HFD	High-fat diet
MST	Microscale thermophoresis
OCT1	Organic cation transporter-1
pAMP	Plasma 5'-AMP
PEG-ADA	Polyethylene glycol-modified adenosine deaminase
pH _i	Intracellular pH
PTP1B	Protein tyrosine phosphatase 1B
STZ	Streptozotocin

Research in context

What is already known about this subject?

- Plasma 5'-AMP (pAMP) is elevated in the *db/db* mouse model of diabetes
- The injection of 5'-AMP causes hyperglycaemia in wild-type mice
- Protein tyrosine phosphatase 1B (PTP1B) negatively regulates insulin receptor phosphorylation

What is the key question?

- How do adenine nucleotides induce hyperglycaemia in type 2 diabetes?

What are the new findings?

- The elevation of pAMP was required for the occurrence of hyperglycaemia in type 2 diabetes in mouse models and in humans
- Adenine nucleotide metabolites inhibited Na^+/K^+ ATPase and Na^+/H^+ exchanger activity
- ATP accumulation changed the cellular pH homeostasis in the liver, directly stimulating PTP1B activity

How might this impact on clinical practice in the foreseeable future?

- Adenine nucleotide-related intracellular pH is a potential therapeutic target in obesity and type 2 diabetes

Introduction

Endogenous glucose production, including hepatic glycogenolysis and gluconeogenesis, is essential for survival during starvation [1]. On the other hand, excessive glucose production contributes to hyperglycaemia in individuals with diabetes [2]. In type 2 diabetes, hyperglycaemia occurs due to insulin insensitivity in main metabolic organs such as liver and skeletal muscle [3]. Obesity is a major risk factor for type 2 diabetes [4]. Mice deficient in leptin and leptin receptors (*ob/ob* and *db/db*) exhibit severe, spontaneous, early-onset obesity, with a body weight three times that of wild-type mice [5], and display type 2 diabetes-like characteristics such as insulin insensitivity, glucose intolerance and hyperinsulinaemia [6]. Both *db/db* and *ob/ob* mice develop hyperglycaemia after the age of 4 weeks [7, 8]. However, with increasing age, blood glucose levels of *ob/ob* mice become nearly normal even in the presence of obesity and severe insulin resistance [8, 9]. Similarly, most high-fat diet (HFD)-induced obese mice maintain non-diabetic glucose levels and present insulin resistance only [10]. In humans, although most people who develop type 2 diabetes are overweight or obese, most obese individuals do not become diabetic even though they present with a high degree of insulin resistance [4].

The failure of pancreatic beta cells to compensate for insulin resistance is thought to release the suppression of gluconeogenesis in the liver and cause hyperglycaemia [11]. However, it is not possible to determine separately the effect of hyperglycaemia and dyslipidaemia on beta cell

differentiation status in diabetic mice or in pathological samples from human donors with diabetes [12]. Whether additional factors are acquired secondary to changes in the metabolic milieu associated with the progression to type 2 diabetes remains elusive. It is well known that insulin receptor tyrosine kinase is directly involved in intracellular insulin signalling transduction [13] and that protein tyrosine phosphatase 1B (PTP1B) negatively regulates insulin receptor phosphorylation [14]. It has been observed that the activity of insulin receptor tyrosine kinase does not change in *db/db* diabetic mice [15]. Inhibition of PTP1B is demonstrated to improve insulin sensitivity in obese and insulin-resistant *ob/ob* mice and reduce the hyperglycaemia in obese *db/db* mice [16].

Metformin is used extensively to treat type 2 diabetes. It acts through multiple mechanisms such as increasing phosphorylation of insulin receptors, stimulating translocation of GLUT4 to the cell membrane, decreasing gluconeogenesis in hepatocytes and activation of AMP-activated protein kinase (AMPK) [17–20]. Other research indicates metformin inhibits the respiratory chain that restrains hepatic gluconeogenesis [21].

Our previous observation indicates that plasma 5'-AMP (pAMP) was elevated in plasma of obese *db/db* diabetic mice and that exogenous 5'-AMP caused hyperglycaemia-like type 2 diabetes in wild-type mice [22]. We are interested in understanding the role of adenine nucleotides in metabolic regulation, insulin resistance and hyperglycaemia occurring in type 2 diabetes. In this study, we have investigated the role of adenine nucleotides in regulating the activity of PTP1B in

type 2 diabetes. Our studies provide insight into the participation of adenine nucleotides in obese diabetic mice.

Methods

Animal models Mice were obtained from SLACCAS (Shanghai, China). Male C57BL/6, C57BL/6 *ob/ob*, C57BL/Ks *db/db* mice and their homozygous lean littermates (+/+) were used. To create diet-induced insulin resistance models, 4-week-old male C57BL/6 mice were fed an HFD. HFD-fed mice were injected intraperitoneally with streptozotocin (STZ) to induce diabetes. All procedures were approved by the Animal Care and Use Committee at Nanjing University of Science and Technology (NJUST-AC-05-1217). See electronic supplementary material (ESM) Methods for further details.

Injection of 5'-AMP and metformin and ITT Male 8-week-old C57BL/6 mice were injected intraperitoneally with 5'-AMP at 09:00. Metformin hydrochloride was administered by gavage. Mice were killed 1 h after 5'-AMP treatment. For ITT, mice were fasted and injected intraperitoneally with insulin. See ESM Methods for details.

Tissue and blood sampling Mice were euthanised, blood was collected and then tissues were removed and freeze-clamped in liquid nitrogen. Erythrocytes and plasma were separated. See ESM Methods for further details.

HPLC analysis of nucleotides and metabolites Mouse livers were homogenised using ice-cold 0.4 mol/l perchloric acid. Samples were analysed using HPLC (Waters 1525 System; Millipore, Bedford, MA, USA) on a reversed-phase C18 column as described previously [23–25]. Pure nucleotides and metabolites were used to identify the peaks and obtain the calibration curves. Quantification was based on the area under the peaks.

¹H NMR-based metabolomics analysis of tissue extraction Tissues were extracted using ice-cold 50% acetonitrile. The spectra were acquired on a Bruker AV 500-MHz spectrometer (Bruker, Karlsruhe, Germany). The integrated area was normalised. Unsupervised principal component analysis methods were performed. According to the integrated areas of metabolites, the increased or decreased factors were calculated. See ESM Methods for details.

Na⁺/K⁺ ATPase assay for hepatic plasma membrane Liver plasma membrane was prepared as described previously [26]. Na⁺/K⁺ ATPase assay was performed as previously described, with modification [27]. The reaction was started by adding ATP. Released inorganic phosphate was measured. Na⁺/K⁺ ATPase activity was calculated by comparison with

the ATPase activity inhibited by ouabain. See ESM Methods for details.

Quantitative real-time PCR Total RNA from mouse liver was isolated and quantitative real-time PCR was used to determine relative mRNA of mouse *Pepck* (also known as *Pck1*) and *G6pase* (also known as *G6pc*). See ESM Methods for details; primers are listed in ESM Table 1.

Glucose-6-phosphatase and PEPCK activity measurement Glucose-6-phosphatase (G6Pase) and PEPCK activity were analysed as previously described [28, 29]. See ESM Methods for details.

Treatment of *db/db* mice with polyethylene glycol-modified adenosine deaminase Polyethylene glycol-modified adenosine deaminase (PEG-ADA) was prepared with recombinant adenosine deaminase and mPEG-succinimidyl propionate (mPEG-SPA; Chemgen Pharma, Beijing, China). Obese *db/db* mice were injected with PEG-ADA prior to experiments. See ESM Methods for details.

Preparation of metformin Metformin hydrochloride (20 g) was dissolved in 1 litre of methanol containing 2% vol./vol. water. The solution was then passed through a column containing anion exchange resin Duolite A101D (Jinjinle Chemical Co., Changsha, China). The column was then rinsed with 1 litre of methanol containing 2% vol./vol. water. The elute solution was evaporated to dryness under reduced pressure at 40°C to obtain metformin, which was used immediately.

PTP1B activity and kinetic analysis PTP1B activity in liver extract was determined as described previously, with modification [30]. Liver was homogenised in cold PBS. The supernatant fractions were diluted and reaction was started by adding p-nitrophenyl phosphate. For kinetic analysis, recombinant PTP1B was used. See ESM Methods for details.

Microscale thermophoresis binding analyses Microscale thermophoresis (MST) analysis was performed using the MST device (Monolith NT.115) as described previously with modification [31]. Data were treated with NT analysis software (see ESM Methods for further details).

Intracellular pH measurement The measurement of intracellular pH (pH_i) was performed as previously described, with modification [32]. AML12 cells were used and stained with Hanks' buffered saline solution (HBSS) solution containing 1 μmol/l (2',7'-bis-[carboxyethyl]-5-[and-6]-carboxyfluorescein)-tetraacetoxymethyl ester (BCECF-AM) for 20 min at room temperature. Then the cells were perfused with HBSS. pH_i measurements were performed at 37°C using a fluorescence microscope (Nikon, Tokyo, Japan). BCECF

fluorescence was excited at 490 nm and 440 nm and emitted fluorescence was measured at 530 nm. The F490/F440 emission ratio was converted to pH scale according to the nigericin technique. See ESM Methods for details.

Na⁺/H⁺ exchanger activity Na⁺/H⁺ exchanger activity was analysed in bicarbonate-free solution. After equilibrating, AML12 cells were acidified by adding propionate. The recovery rate of pH_i was calculated from data obtained during the initial recovery period. See ESM Methods for details.

Intracellular sodium determination AML12 cells were treated with 5'-AMP. After removal of medium, cells were washed and lysed. Intracellular sodium was estimated using atomic absorption spectrometry. See ESM Methods for details.

Vacuolar-type (H⁺) ATPase activity The microsome fraction was prepared from mouse liver by differential centrifugation. Hydrolysis of ATP by microsome was analysed. Released inorganic phosphate was measured. V-ATPase activity was defined as the ATPase activity inhibited by bafilomycin A1. See ESM Methods for details.

Study participants Twenty-five individuals with type 2 diabetes and 21 healthy individuals (control group) were recruited. Blood samples were obtained in the early morning through the Second Hospital of Nanjing. Upon arrival, body mass and height measurements were taken to determine BMI, and blood samples were obtained using venepuncture. All blood samples were obtained after informed consent was obtained from both the diabetic and healthy individuals for their participation in the study. HbA_{1c} was determined with the gold standard ion-exchange method (Tosoh G8 HPLC Analyzer, Tosoh Bioscience, San Francisco, CA, USA). Blood glucose was determined using a OneTouch Ultra blood glucose meter (Life-Scan, Milpitas, CA, USA). Plasma insulin was analysed using the Roche E170 chemiluminescent immunoassay (Roche Diagnostics). Plasma was deproteinised using ice cold 0.4 mol/l perchloric acid and then analysed using HPLC. Demographics are shown in Table 1. All procedures were approved by the Institutional Review Board of Nanjing University of Science and Technology.

Statistical analysis Data are presented as means ± SD. Statistical analysis was performed by Student's *t* test, one-way ANOVA or two-way ANOVA followed by Tukey's post hoc test. Significance was defined as *p* < 0.05.

Results

Elevated pAMP as a biomarker for type 2 diabetes in mouse models To investigate whether elevated pAMP is an independent event in obese *db/db* diabetic mice, we measured pAMP

Table 1 Participant demographics

Characteristic	Healthy	Type 2 diabetes
<i>n</i> (women/men)	25 (10/15)	21 (9/12)
Age (years)	35 ± 10	40 ± 11
Diabetes duration (<1/<10/≥10 years)	-	3/8/10
Body weight (kg)	63.2 ± 15.1	78.3 ± 12.1**
BMI (kg/m ²)	21.5 ± 4.8	29.4 ± 3.7**
HbA _{1c} (mmol/mol)	-	64.5 ± 16.9
HbA _{1c} (%)	-	8.1 ± 1.5

Data are mean ± SD

***p* < 0.01 vs healthy participants (Student's *t* test)

levels in different mouse models of insulin resistance. In accordance with findings from a previous study, pAMP increased in *db/db* vs lean mice (13.9 ± 2.3 μmol/l vs 3.7 ± 0.9 μmol/l; *p* < 0.01; Fig. 1a). The increase in pAMP was also observed in diabetic *ob/ob* vs lean mice (9.1 ± 2.0 μmol/l vs 3.9 ± 1.2 μmol/l; *p* < 0.01; Fig. 1b), HFD-STZ mice vs wild-type mice (6.6 ± 1.5 μmol/l vs 4.1 ± 0.9 μmol/l; *p* < 0.05; Fig. 1c) and wild-type mice treated with 5'-AMP vs saline (154 mmol/l NaCl) (15.1 ± 2.1 μmol/l vs 4.4 ± 1.5 μmol/l; *p* < 0.01; Fig. 1d). Then we analysed the pAMP level in non-diabetic mice with insulin resistance. The results showed no difference in pAMP level between non-diabetic *ob/ob* and non-diabetic HFD mice compared with control mice (Fig. 1e,f). To understand the role of increased pAMP in metabolic regulation in type 2 diabetes, a survey of ¹H NMR-based liver metabolomic data was performed in an obese *db/db* diabetic mouse model for insulin resistance and metabolic disorders (ESM Fig. 1). The plots generated from profiles of extracts of the insulin-sensitive liver in lean and *db/db* obese mice revealed significant separation of metabolites between the two groups (Fig. 1g,h), suggesting that these phenotypes exhibit significantly different metabolic patterns of known and unknown metabolites (ESM Table 2). Interestingly, when we injected 5'-AMP into the wild-type mice, the profiles of liver extracts in these mice exhibited similar metabolomic shifts to those observed in the *db/db* mice (Fig. 1i,j; ESM Table 2). A heat map of the 38 indicated metabolites in at least one of three pair-wise comparisons is shown in Fig. 1k. Pearson *r* analysis showed strong correlations between biological replicates in each group. Metabolic pathway analysis of the differential metabolite clusters revealed that livers of 5'-AMP-treated and *db/db* mice are enriched in metabolite accumulations including amine acid and nucleotide metabolism. Together, these results suggest that elevated pAMP is a common marker and plays a potential role in hyperglycaemia in mouse models of type 2 diabetes.

The accumulation of liver ATP and adenine nucleotide metabolites is required for hyperglycaemia in obese mice HPLC

Fig. 1 pAMP is elevated in mouse models of type 2 diabetes. (**a–d**) HPLC analysis of pAMP in mouse models of type 2 diabetes. pAMP levels, measured 1 h after 5'-AMP treatment, were significantly increased in *db/db* (**a**; $n = 5$), diabetic *ob/ob* (**b**; $n = 5$), HFD-STZ (**c**; $n = 5$) and 5'-AMP-treated (**d**; $n = 5$) mice vs the respective control groups. (**e, f**) HPLC analysis of pAMP in non-diabetic insulin resistance models. pAMP in non-diabetic *ob/ob* (**e**; $n = 6$) and non-diabetic HFD (**f**; $n = 5$) mice did not differ significantly from control groups. (**g–j**) Metabolomics analysis

based on ^1H NMR data from livers of lean and *db/db* mouse groups (**g, h**) and the 5'-AMP-treated and saline-treated control groups (**i, j**). (**k**) Heat map of 38 metabolites identified in at least one of three comparisons. In (**g–k**) $n = 6$ per group. Refer to ESM Fig. 1 for numbered metabolites in (**h, j**). Data are expressed as mean $\pm$ SD; * $p < 0.05$ and ** $p < 0.01$ compared with the respective control group (Student's t test). Ctrl, control; PCA, principal component analysis; PC1/2, principal components 1 and 2

analysis for the detection of water-soluble liver chemicals confirmed that, relative to lean mice, *db/db* mice exhibited significantly elevated ATP and adenine nucleotide metabolites (ANMs), including adenosine, hypoxanthine, xanthine and uric acid in the livers (Fig. 2a,b). Similarly, ATP and ANMs were also increased in the liver of 5'-AMP-treated mice compared with saline-treated control mice (Fig. 2c,d). These results indicate that abnormal adenine nucleotide metabolism occurs and liver ATP and ANMs accumulate in obese diabetic *db/db* mice. Next, we investigated whether abnormal adenine nucleotide metabolism occurs in other mouse models of diabetes. As observed in *db/db* diabetic mice, HPLC analysis showed obvious elevation of ATP and ANMs in the liver of the obese diabetic *ob/ob* mice (ESM Table 3), but not in non-diabetic obese *ob/ob* mice even in the presence of severe insulin resistance. In another high-fat STZ-induced type 2 diabetes model, we found elevated ATP and ANMs in the liver of hyperglycaemic mice but no accumulation of ATP and ANMs in liver in mice with HFD-induced obesity (ESM Table 3); the latter mice exhibited insulin resistance but had normal blood glucose levels. Interestingly, adenosine increased in the liver of all obese

mice and ATP and ANMs were only elevated in the liver of hyperglycaemic mice. To investigate whether the accumulation of ANMs and ATP contributed to hyperglycaemia, we injected 5'-AMP into the wild-type mice and examined the time course of induction of hyperglycaemia and the changes in liver concentrations of ATP and ANMs. Injection of 5'-AMP induced hyperglycaemia in 0.5–1 h and the blood glucose levels returned to normal after 2 h (Fig. 2e). HPLC analysis revealed that ANMs and ATP levels in liver increased at 1 h and returned to baseline levels at 2 h after 5'-AMP injection (Fig. 2f,g). These observations suggest that the accumulation of ATP and ANMs in liver is required for the occurrence of hyperglycaemia in obese mice. Next, we investigated the potential relationship between cellular ATP and ANMs. Figure 2h–k shows the in vitro effects of ANMs on Na^+/K^+ ATPase activity in plasma membrane in mouse liver. Adenosine had no effect on Na^+/K^+ ATPase activity but ANMs such as hypoxanthine, xanthine and uric acid markedly inhibited Na^+/K^+ ATPase activity in a dose-dependent manner. In most animal cells, Na^+/K^+ ATPase is responsible for about one-fifth to two-thirds of the cell's energy expenditure [33]. We reasoned that impairment of Na^+/K^+ ATPase

Fig. 2 Hyperglycaemia in obese mice requires accumulation of ATP and ANMs in the liver. (**a**, **b**) Quantification of adenine nucleotides (**a**) and ANMs (**b**), including adenosine (Ado), hypoxanthine (Hyp), xanthine (Xan) and uric acid (UA), in the liver of *db/db* mice compared with lean mice. In *db/db* mice, ATP and ANMs are elevated in the liver ($n = 5$). (**c**, **d**) Quantification of adenine nucleotides (**c**) and ANMs (**d**) in the liver of 5'-AMP-treated wild-type mice compared with saline-treated control mice. Similarly, in the 5'-AMP-treated mice, ATP and ANMs are increased in the liver ($n = 5$). (**e**) The time course of 5'-AMP-induced

hyperglycaemia in wild-type mice ($n = 6$). (**f**, **g**) Quantification of adenine nucleotides and ANMs in the livers of 5'-AMP-treated mice compared with saline-treated control mice ($n = 5$). (**h**–**k**) Na⁺/K⁺ ATPase activity, expressed as fold vs no treatment, was decreased in the presence of ANMs, including Hyp (**i**; $n = 7$), Xan (**j**; $n = 7$) and UA (**k**; $n = 5$) but not Ado (**h**; $n = 6$). Data are expressed mean $\pm$ SD. Statistical analysis was performed with Student's *t* test (**a**–**g**) or one-way ANOVA and Tukey's post hoc test (**h**–**k**); * $p < 0.05$ and ** $p < 0.01$ compared with the control group; † $p < 0.05$ compared with 1 h group

activity by ANMs should decrease ATP utilisation and cause a cellular ATP elevation in liver.

Metformin lowers 5'-AMP induced hyperglycaemia and decreases cellular ATP level Metformin, a widely used glucose-lowering drug, markedly alleviated 5'-AMP induced hyperglycaemia (Fig. 3a,b). Our analysis showed that metformin decreased 5'-AMP-induced *G6Pase* and *Pepck* mRNA expression levels (Fig. 3c). Metformin also reduced 5'-AMP-related increases in *G6Pase* and *PEPCK* activity (Fig. 3d). Moreover, metformin had no effect on the activity of Na⁺/K⁺ ATPase (ESM Fig. 2). HPLC analysis revealed that metformin decreased 5'-AMP-induced ATP elevation but failed to change 5'-AMP-induced ANM accumulation in the liver (Fig. 3e,f). Similar results were also observed in the metformin-treated *db/db* mice (Fig. 3g,h). These results suggest that elevated liver ATP plays a potential role in inducing hyperglycaemia.

ATP is an activator of PTP1B One key question arising from these studies is the biological significance of this abnormal

purinergic metabolism in mice. Perhaps increased ATP level in the liver of obese mice is an important signal for energy utilisation related to glucose metabolism. Our previous work demonstrates that adenosine is an ATP-competitive substrate inhibitor in the phosphorylation of insulin receptors in vitro [34]. We tested whether decreasing cellular adenosine levels alleviated hyperglycaemia in obese *db/db* mice. PEG-ADA was injected intraperitoneally into *db/db* mice to decrease liver adenosine levels. As expected, PEG-ADA obviously decreased cellular adenosine level in mouse livers (ESM Fig. 3). However, PEG-ADA failed to lower blood glucose level in *db/db* mice (ESM Fig. 3), suggesting that adenosine is not a key regulator of blood glucose in vivo. An unexpected finding was that ATP dose-dependently enhanced the activity of PTP1B, a known negative regulator of the insulin signalling pathway (Fig. 4a). To rule out the possibility that this was a non-specific effect that could be triggered by any nucleotide, we added various concentrations of AMP, adenosine and uric acid to mouse liver extracts but none of these molecules increased PTP1B activity (Fig. 4b). A kinetics study of the stimulatory effects of ATP on recombinant PTP1B activity was

Fig. 3 Metformin reduces hyperglycaemia in 5'-AMP-treated mice and *db/db* mice. (**a**, **b**) Metformin (MET) lowers 5'-AMP-induced hyperglycaemia (**a**) and improves 5'-AMP-induced insulin insensitivity as measured by ITT (**b**) in wild-type mice. (**c**) Relative gene expression of *G6Pase* and *Pepck* in wild-type mouse liver. Metformin decreased 5'-AMP-activated *G6Pase* and *Pepck* mRNA levels in wild-type mice. Using β -actin as internal control, data were analysed according to the $2^{-\Delta\Delta C_t}$ method. The expression of all genes is shown as fold change relative to saline-treated control group. (**d**) Metformin ameliorated 5'-

AMP-related increases in *G6Pase* and *PEPCK* activity in the liver of wild-type mice. (**e**, **f**) Quantification of liver nucleotides and ANMs, including adenosine (Ado), hypoxanthine (Hyp), xanthine (Xan) and uric acid (UA), in 5'-AMP-, metformin- and saline-treated wild-type mice. (**g**, **h**) Quantification of liver nucleotides and ANMs in metformin-treated *db/db* mice, saline-treated *db/db* mice and control lean mice ($n = 5$ per group). Data are expressed mean $\pm$ SD; * $p < 0.05$ and ** $p < 0.01$ vs saline-treated or lean control group; † $p < 0.05$ and †† $p < 0.01$ for metformin vs 5'-AMP-treatment or *db/db* group (Student's *t* test)

performed over the substrate concentration range of 0.25–2 mmol/l in the presence of 0.2 or 0.4 mmol/l of ATP. Lineweaver–Burk double-reciprocal plot analysis revealed that ATP increased the maximal PTP1B velocity and decreased the K_m value. The K_i value (activation constant) obtained from the Dixon plot was 0.65 mmol/l (Fig. 4c–e). These studies demonstrate that ATP is an activator of PTP1B and can function as a negative feedback regulator of the insulin signalling pathway.

Metformin is an inhibitor of PTP1B We next investigated whether the stimulatory effect of ATP on PTP1B activity can be inhibited by glucose-lowering drugs such as metformin. Because metformin must be transported into the cell as cationic metformin [35], we first assayed PTP1B activity with cationic metformin in vitro, and found that cationic metformin could effectively inhibit the ATP-stimulated activity of recombinant PTP1B (Fig. 4f,g). Cationic metformin also decreased the activity of the PTP1B in mouse liver extracts in a dose-dependent manner (Fig. 4h). In contrast to the effect of ATP, cationic metformin decreased the maximal PTP1B velocity and increased its K_m value based on Lineweaver–Burk double-reciprocal plot analysis (Fig. 4i–k). The K_i value (inhibition constant) obtained from the Dixon plot was 0.31 mmol/l. These results indicate that cationic metformin is an inhibitor of PTP1B.

A reciprocal regulation of PTP1B activity by ATP and metformin To further understand the effects of ATP and metformin on PTP1B activity, we investigated the interaction between

PTP1B and ATP or metformin by MST assay. The fluorescence intensity of labelled PTP1B detected by the MST reader did not vary until micromolar levels of ATP or cationic metformin were present (Fig. 5a–d). This suggests a lower affinity between ATP and PTP1B. Similarly, metformin displayed a lower affinity for PTP1B. The sigmoidal binding curve observed in the presence of ATP was different from that obtained with metformin. The different shape of the fit curves can be related to pH-dependent thermophoresis type [36]. Next, we observed that the binding curve obtained at acidic pH values was equal to that following addition of ATP and that the binding curve obtained at basic pH values was similar to that following addition of metformin (Fig. 5e–h). These data suggest that ATP and metformin might influence pH value, resulting in the variation in fluorescence intensity under the MST reader. Indeed, a precise pH assay revealed that ATP and metformin caused a reciprocal change in the pH of assay buffers (Fig. 5i,j) and that PTP1B activity was strongly dependent on pH (Fig. 5k).

Extracellular nucleotides and metformin regulate pH_i homeostasis in hepatocytes It is known that uptake of metformin hydrochloride into hepatocytes is catalysed by the organic cation transporter-1 (OCT1) [35]. Being positively charged, the cation metformin accumulates in cells [21]. We investigated whether exogenous metformin hydrochloride and nucleotides induced intracellular pH changes in cultured hepatocytes. After addition of metformin hydrochloride and ATP, hepatic pH_i was measured from green (BCECF) images taken at 490 nm and 440 nm excitation wavelengths. Under control

Fig. 4 Reciprocal regulation of hepatic PTP1B activity by 5'-AMP and metformin. **(a)** ATP increased PTP1B activity in liver extracts from C57BL/6 mice ($n = 6$). **(b)** ATP metabolites, including 5'-AMP (AMP), adenosine (Ado) and uric acid (UA), had no obvious effect on PTP1B activity in liver extracts ($n = 3$). **(c–e)** Recombinant PTP1B stimulation was analysed in the presence of different concentrations of ATP (mmol/l) ($n = 3$). ATP stimulated the activity of PTP1B **(c)**. Lineweaver–Burk plot for PTP1B stimulation by ATP indicated that ATP increased the $V_{\max}$ of PTP1B and decreased the K_m of PTP1B substrate **(d)**. The activation constant was determined by interpretation of the Dixon plot **(e)**. **(f, g)** Cationic metformin (MET) (mmol/l) **(g)**, not metformin hydrochloride (MET HCl) (mmol/l) **(f)**, inhibits ATP-stimulated (mmol/l) recombinant PTP1B activity ($n = 3$). **(h)** Cationic metformin inhibits PTP1B activity in a dose-dependent manner in liver extracts ($n = 4$). **(i–k)** Recombinant

PTP1B stimulation was analysed in the presence of different concentrations of metformin (mmol/l) ($n = 3$). Metformin inhibited the activity of PTP1B **(i)**. Lineweaver–Burk plots for recombinant PTP1B inhibition by metformin indicates that metformin decreased the $V_{\max}$ of PTP1B and increased the K_m value for the PTP1B substrate **(j)**. The inhibition constant was determined by interpretation of the Dixon plots **(k)**. The range for substrate pNpp concentrations is 0.25–2 mmol/l in **(c, f, g, i)** and 0.25–1 mmol/l in **(d, e, j, k)**. The results represent the mean $\pm$ SD. Data in **(a, b, h)** are expressed as fold vs 0 mmol/l metformin. Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test **(a, b, h)** or by two-way ANOVA followed by Tukey's post hoc test **(c, f, g, i)**. * $p < 0.05$ and ** $p < 0.01$ vs control group. Δ Abs, change in absorbance; pNpp, p-nitrophenyl phosphate

conditions, the typical effect of extracellular 5'-AMP on pH_i in hepatocytes is illustrated in Fig. 6a. Exposure to 5'-AMP resulted in a rapid decrease of 0.45 ± 0.08 pH units; lower concentrations of 5'-AMP (10 μ mol/l and 100 μ mol/l) also

reduced intracellular pH to different degrees (ESM Fig. 4). Extracellular metformin hydrochloride caused pH_i to rise (Fig. 6b). Cytoplasmic pH was stable at 7.2–7.4 under the control experimental conditions but promptly became acidic

Fig. 5 Interaction between ATP, metformin and PTP1B. **(a–d)** MST data analysis of ATP **(a, b)** and cationic metformin (MET) **(c, d)** with PTP1B. The binding curve observed in the presence of ATP was different from that obtained with metformin. **(e–h)** MST data analysis of HCl **(e, f)** and NaOH **(g, h)** with fluorescently labelled recombinant PTP1B. The binding curves in **(e)** and **(g)** were similar to those observed for ATP **(a)** and cationic metformin **(c)**, respectively. Traces in **(a)**, **(c)**, **(e)** and **(g)** represented the relative fluorescence over time of different ligand concentrations. The red traces correspond to the point of highest concentration

shown in **(b)**, **(d)**, **(f)** and **(h)**, respectively. The F_{norm} value was calculated by taking the ratio between the relative fluorescence after heating (infrared [IR] laser off) and before heating (IR laser on), as shown for **(a)**. **(i, j)** The effects of ATP **(i)** and metformin **(j)** on pH of PTP1B assay buffers. **(k)** Effects of buffer pH on recombinant PTP1B activity, expressed as activity relative to activity at pH 7.4. For **(a–j)**, results are representative of three independent experiments. Data in **(k)** are shown as mean $\pm$ SD of three samples; * $p < 0.05$ and ** $p < 0.01$ vs activity at pH 7.4 (one-way ANOVA followed by Tukey's post hoc test)

(6.7–6.9) after 5'-AMP treatment. On the contrary, treating cells with metformin hydrochloride increased the cytoplasmic pH to 7.5–7.8. Representative wide-field fluorescence images of the BCECF green fluorescence excited at two wavelengths, together with a pseudocoloured ratio image map of cell pH, are shown in Fig. 6c. Next, we found that ANMs, including hypoxanthine, xanthine and uric acid, significantly reduced Na^+/H^+ exchanger activity in AML12 cells (ESM Fig. 5), indicating that the ANMs caused deregulation of pH_i . A decreased intracellular Na^+ level was observed in 5'-AMP-treated cells (ESM Fig. 6). The ANMs had no effects on liver vacuolar H^+ ATPase activity (ESM Fig. 7). Together, our studies suggest that accumulated ANMs inhibit Na^+/H^+ exchanger activity and that ATP and metformin, as proton donors and receptors, reciprocally regulate pH_i homeostasis in liver cells (Fig. 6d).

pAMP was elevated in people with type 2 diabetes To further investigate the potential association between elevated pAMP and type 2 diabetes, we analysed the pAMP level in individuals with type 2 diabetes. The clinical characteristics of the

participants are shown in Table 1. Twenty-five participants with type 2 diabetes and 21 non-diabetic healthy control individuals participated in the study. The control group had similar age and sex distribution to the diabetes patients. Diabetic individuals had higher BMI and HbA_{1c} . As shown in Fig. 7a, although the absolute level of pAMP in humans was far less than that in mice, it was still significantly elevated in diabetic individuals (45.2 ± 22.7 nmol/l) compared with non-diabetic individuals (3.1 ± 1.9 nmol/l). Fasting plasma glucose and insulin concentrations were significantly increased in the diabetic individuals (Fig. 7b,c). Together, these observations confirm that elevated pAMP is a universal marker in type 2 diabetes.

Discussion

Our results show that ATP and ANM accumulation in liver cells promote hyperglycaemia in obesity-related type 2 diabetes. Sustained hyperglycaemia impairs insulin-stimulated glucose utilisation in the skeletal muscle, a phenomenon that is referred

Fig. 6 Effects of exogenous 5'-AMP and metformin on pH_i of AML12 hepatocytes. **(a)** 5'-AMP produced a rapid decrease in pH_i . **(b)** Extracellular metformin hydrochloride (MET HCl) increased pH_i . **(c)** Fluorescence images of BCECF-stained AML12 hepatocytes taken at 440 nm and 490 nm excitation wavelengths. The pseudocoloured ratio image shows a spatial map of pH_i (colour pH scale to the right). The images show the difference of pH_i between cells treated with 5'-AMP or metformin hydrochloride and the control group (Ctrl). **(d)** Model showing the pivotal role of cellular pH in regulation of insulin negative

signalling of PTP1B involving ATP and its metabolites. NEFA-related increase in intracellular ANM accumulation inhibits Na^+/K^+ ATPase and Na^+/H^+ exchanger activity, resulting in cellular ATP elevation and pH_i deregulation. Cellular ATP donates H^+ , directly causing cytoplasmic acidification, and activates the insulin negative regulatory pathway of PTP1B, increasing hepatic glucose production (via PEPCK and G6Pase). Metformin elevates hepatic pH_i and substantially increases insulin sensitivity. Ado, adenosine; Hyp, hypoxanthine; IR, insulin receptor; P, phosphate; UA, uric acid; Xan, xanthine

to clinically as glucose toxicity. Although insulin resistance is thought to arise during the early stage of type 2 diabetes, this process often remains undetectable. In contrast to hyperglycaemia, insulin resistance is a well-evolved protective mechanism during acute illness that helps conserve the brain's glucose supply by preventing muscles from taking up excessive glucose [37]. Thus, a better understanding of the pathological causes of hyperglycaemia in type 2 diabetes is essential for more rational and appropriate treatment of diabetic individuals.

Release of NEFA from adipose tissue and increased levels of ANMs such as uric acid are important hallmarks of insulin resistance and type 2 diabetes [38, 39]. NEFA-induced nucleotide release from vascular endothelial cells is important for accumulation of ANMs in organs such as liver and skeletal muscle [22]. Inhibiting ATP release by drugs such as glibenclamide (known as

glyburide in the USA and Canada) improves insulin resistance and decreases blood glucose levels in type 2 diabetes [40]. It is reported that insulin resistance develops within hours of an acute increase in plasma NEFA levels in humans [41], and hepatic inflammation has been suggested to be a consequence (rather than a cause) of insulin resistance [42]. Thus, elevated levels of plasma nucleotides could serve as an early signal for diabetes. Different from diabetic mice, non-diabetic obese mice did not display a significant change in pAMP level. Non-diabetic *ob/ob* mice had higher NEFA levels compared with lean mice but the levels were much lower than in diabetic *db/db* mice [43]. Thus, levels of nucleotides released by NEFA in these obese mice might be less than in diabetic mice. Ecto-5'-nucleotidase in circulation converts nucleotides to adenosine and is rapidly transported into cells [44]. The intracellular effect of plasma nucleotides is likely mediated through increased cellular adenosine levels. Indeed, adenosine is an inhibitor of insulin signalling [34]. However, elevation of adenosine alone causes insulin resistance but not hyperglycaemia, as observed in *ob/ob* and HFD-fed mice. Similarly, a low dose of 5'-AMP induced insulin resistance rather than hyperglycaemia. With increased plasma nucleotides, liver accumulates ANMs and hyperglycaemia occurs. ANMs are independent inhibitors of Na^+/K^+ ATPase; this inhibition inevitably reduces ATP utilisation, resulting in elevation of hepatic ATP. Cellular ATP elevations were also observed in diabetic or 5'-AMP-treated mouse kidney but not skeletal muscle (ESM Fig. 8). On the other hand, ANMs also inhibited activity of the Na^+/H^+ exchanger, which is crucial in maintaining homeostasis of pH_i and sodium [45]. Thus, ANM accumulation could cause a deregulation of pH_i .

Fig. 7 pAMP was elevated in individuals with type 2 diabetes. Quantification of pAMP by HPLC analysis **(a)**, fasting blood glucose **(b)** and fasting plasma insulin **(c)** in individuals with type 2 diabetes. The diabetic individuals had elevated pAMP levels compared with a control group of healthy individuals. Values are shown as mean $\pm$ SD, $n = 21$ (healthy) and $n = 25$ (diabetic). * $p < 0.05$ and ** $p < 0.01$ vs healthy individuals (Student's t test)

Coincidentally, pH is an important regulatory factor for PTP1B [46], which negatively regulates insulin signalling by dephosphorylating tyrosine residues on insulin receptors and IRS proteins [14]. Loss of PTP1B function in mice is associated with increased insulin sensitivity [47]. In our liver cell extracts or recombinant PTP1B enzyme assay system, ATP enhanced PTP1B activity in a dose-dependent manner; metformin (but not metformin hydrochloride) caused a dose-dependent inhibition of PTP1B activity. ATP and metformin acted as an activator and an inhibitor of PTP1B, respectively, causing a reciprocal change in the pH of PTP1B assay buffer. This phenomenon was further proved by binding assay. Metformin, an oral glucose-lowering agent that is widely used in the management of non-insulin-dependent diabetes mellitus [48], significantly improved nucleotide-induced insulin resistance and hyperglycaemia. Metformin is primarily excreted unchanged in the urine, with negligible metabolism [49], and is a substrate for OCT1 [39], which is responsible for the hepatic uptake of metformin. Thus, metformin hydrochloride must be transported into cells in the form of cationic metformin. Indeed, the cation metformin is observed to accumulate in cells [21]. Metformin is a stronger base than most other basic drugs [49]. Therefore, it is believed that hepatic uptake of cationic metformin is an essential step in elevating hepatic cell pH, as well as the occurrence of PTP1B-inhibiting effects such as improved insulin sensitivity.

Thus, our studies uncover a novel key step in the pathogenesis of diabetes-related excessive hepatic glucose production and reveal an emerging role for adenine nucleotides that permits hyperglycaemia to occur in obese mice. As illustrated in Fig. 6d, NEFA-related increase in intracellular ANM accumulation inhibits Na^+/K^+ ATPase and Na^+/H^+ exchanger activity, resulting in cellular ATP elevation and pH_i deregulation. Cellular ATP, as an H^+ donor, directly causes cytoplasmic acidification and activates the insulin negative regulatory pathway of PTP1B, increasing hepatic glucose production. A causal role of cellular pH homeostasis is shown by the observation that metformin-elevated hepatic pH_i substantially increases insulin sensitivity. More effective pH regulatory agents under development for use in treating obesity-induced insulin resistance may prove worthy of investigation.

Data availability Data are available upon request from the corresponding author.

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Contribution statement XY, YZ and QS performed the experiments. XY, YZ, QS, YXY, YG, WHG and JHL analysed the data, interpreted the results, and reviewed and edited the manuscript. XX, DW and SMW contributed to the discussion, provided advice on experimental design and reviewed the manuscript. JFZ designed the study and wrote the

manuscript. All authors approved the final version of this manuscript. JFZ is the guarantor of this work.

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