Reciprocal regulation of insulin and plasma 5'-AMP in glucose homeostasis in mice

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Abstract

A previous investigation has demonstrated that plasma 5'-AMP (pAMP) exacerbates and causes hyperglycemia in diabetic mice. However, the crosstalk between pAMP and insulin signaling to regulate glucose homeostasis has not been investigated in depth. In this study, we showed that the blood glucose level was more dependent on the ratio of insulin to pAMP than on the absolute level of these two factors. Administration of 5'-AMP significantly attenuated the insulin-stimulated insulin receptor (IR) autophosphorylation in the liver and muscle tissues, resulting in the inhibition of downstream AKT phosphorylation. A docking analysis indicated that adenosine was a potential inhibitor of IR tyrosine kinase. Moreover, the 5'-AMP treatment elevated the ATP level in the pancreas and in the isolated islets, stimulating insulin secretion and increasing the plasma level of insulin. The insulin administration decreased the 5'-AMP-induced hyper-adenosine level by the up-regulation of adenosine kinase activities. Our results indicate that blood glucose homeostasis is reciprocally regulated by pAMP and insulin.

Key Words

- ▶ 5'-AMP
- ▶ insulin
- ▶ hyper-adenosine
- ▶ insulin receptor
- ▶ glucose homeostasis
- ▶ phosphorylation

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Introduction

Type 2 diabetes mellitus is a complex, multisystem disease with a pathophysiology that includes defects in insulin-stimulated peripheral glucose disposal and an impaired suppression of hepatic glucose production (Matthaei *et al.* 2000). Insulin is an essential regulator of intermediary metabolism, and insulin signaling involves a cascade of events initiated by the binding of insulin to insulin receptor (IR) followed by receptor autophosphorylation and activation of receptor tyrosine kinases, which results in tyrosine phosphorylation of the IR substrates (IRSs), including IRS1, IRS2, and IRS3 (Choi & Kim 2010). Binding

of the IRSs to the regulatory subunit of phosphoinositide 3-kinase (PI3K) results in the activation of PI3K, and the PI3K/AKT pathway is a key regulator of insulin signaling (Whiteman *et al.* 2002). In adipose and muscle cells, AKT (AKT1) overexpression results in an increased insulin-sensitive glucose transporter GLUT4 translocation and glucose uptake (Kohn *et al.* 1996, Wang *et al.* 1999). PI3K/AKT signaling also relays IR signals to suppress gluconeogenesis by regulating the gene expression of important enzymes via the phosphorylation-dependent regulation of the Forkhead transcription factor O1 and

the PPAR γ co-activator 1 α (PGC1 α) (Yoon *et al.* 2001, Puigserver *et al.* 2003, Li *et al.* 2007). In diabetic *db/db* mice, insulin-stimulated AKT-Ser⁴⁷³ phosphorylation decreased in the muscle and adipose tissue (Shao *et al.* 2000).

Protein tyrosine phosphatases (PTPs) play a key role in the regulation of reversible tyrosine phosphorylation in the insulin action pathway. Under normal physiological conditions, the phosphorylation of tyrosyl residues on all of the signaling molecules is controlled with a balanced activity between the tyrosine kinases and phosphatases. Tyrosine phosphorylation is the key activator of the IR and IRS proteins, but it is easily reversed either directly or indirectly by the PTPs, such as leukocyte common antigen-related (LAR) phosphatase, PTP1B, 2-SH2-domain-containing protein tyrosine phosphatase 1 (SHP1), and SHP2 (Xu *et al.* 2014). The general reduction in LAR or PTP1B activity enhances insulin-stimulated IR autophosphorylation and downstream substrate activation and signaling (Kulas *et al.* 1995, Egawa *et al.* 2001). The ability of vanadium compounds to inhibit PTPs has been widely used to elucidate the mechanisms of insulin action (Bernier *et al.* 1988, Posner *et al.* 1994), and the effect of vanadate/H₂O₂ (peroxovanadate (PV)) as an inhibitor of PTPs has been reported in a number of studies (Heffetz *et al.* 1992, Ruff *et al.* 1997).

The insulin signaling pathway is activated when nutrients are available, whereas the AMP-activated protein kinase (AMPK) pathway is activated when cells are starved for a carbon source (Hardie 2005). The increase in AMPK activity results in the stimulation of glucose uptake in muscle, fatty acid oxidation, and inhibition of hepatic glucose production, cholesterol and triglyceride synthesis, and lipogenesis (Winder & Hardie 1999, Ruderman *et al.* 2003). The AMPK system acts as a sensor for cellular energy status and is activated by increases in the cellular 5'-AMP and decreases in ATP (Hardie *et al.* 1998). In contrast to insulin, glucagon generally elevates glucose levels in the blood by promoting gluconeogenesis and glycogenolysis. The binding of glucagon to its receptor on the hepatocyte plasma membrane leads to activation of adenylate cyclase, and the production of cAMP increases the hepatic glucose output (Jiang & Zhang 2003). It has been recognized that molecules containing an adenine moiety bind to adenylate cyclase and inhibit its activity (Fain *et al.* 1972).

In our previous study, the elevation in the level of plasma 5'-AMP (pAMP) is a potential upstream regulator of hyperglycemia in type 2 diabetes (Zhang *et al.* 2012). The question as to whether a crosstalk exists between insulin signaling and pAMP remains unknown. In this study, we demonstrate that the blood glucose level is dependent on

the ratio of insulin to 5'-AMP. Exogenous 5'-AMP stimulated insulin release and inhibited the insulin signaling pathway via reducing IR autophosphorylation. Moreover, insulin decreased the 5'-AMP-induced hyper-adenosine level. Our results indicate that a reciprocal regulation of insulin and pAMP maintains glucose homeostasis.

Materials and methods

Animals, antibodies, and chemicals

Male C57BL/6J mice (Comparative Medicine Center, Yangzhou University, China) that were 7–8 weeks old were used in this study. The animals were maintained under 12 h light:12 h darkness cycles with the light on at 0700 h and off at 1900 h and were given food and water *ad libitum*. All of the animal care and use procedures were in accordance with the guidelines provided by the Institutional Animal Care and Use Committee at the Nanjing University of Science and Technology. The anti-IR- β (#3025), anti-phospho-tyrosine (P-Tyr-100) (#9411), anti-AKT (#9272), and anti-phospho-AKT (Ser-473) (#4060) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Adenosine, 5'-AMP, ATP, ADP, insulin, and SQ22536 were purchased from Sigma.

Treatment with 5'-AMP, insulin, and PV

The indicated doses of 5'-AMP or insulin were administered to mice by i.p. injection. The same volume of saline was injected as a control. All of the injections were administered at 0900 h, unless otherwise indicated. After injection, mice were maintained for the desired length of time. The adenylate cyclase inhibitor SQ22536 (10 mg/kg, s.c.) was administered 30 min before the administration of 5'-AMP. Mice were killed by cervical dislocation. Blood was collected from the carotid arteries in anticoagulant tubes containing a stop solution (0.2 mmol/l dipyridamole, 5 mmol/l erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA), and 4.2 mmol/l EDTA), and then the liver, muscle, and pancreas were removed and freeze clamped in liquid nitrogen. The blood samples were immediately centrifuged at 5000 *g* for 5 min at 20 °C. The plasma samples obtained were then stored on ice and immediately used in the experiments. The PV solution was prepared and administered to mice as described previously (Ruff *et al.* 1997). Briefly, a 5 mM solution of sodium vanadate was prepared by heating to boiling (until translucent), and the pH was readjusted to pH 10. Fifteen minutes before use, 30% H₂O₂ was added at room temperature to a final concentration of

50 mM. The PV solutions were injected intraperitoneally into mice at a dose of 10 μ l/g body weight. Twenty minutes after the PV injection, mice were treated with 5'-AMP. Mice were killed by cervical dislocation 30 min after the 5'-AMP injection, and then the liver tissues were removed and freeze clamped in liquid nitrogen.

Immunoprecipitation, western blotting, and IR autophosphorylation analysis

Immunoprecipitation and a western blot assay were performed as described previously (Kruszynska *et al.* 2002). The lysates and immunoprecipitates were resolved by SDS-PAGE, and the proteins were detected by immunoblotting. For the IR autophosphorylation analysis, the liver protein was isolated using cold lysis buffer (Endemann *et al.* 1990). The lysate was incubated for 10 min at room temperature with insulin (2.5 nM) and the indicated doses of adenosine in a 1 ml reaction mixture containing the following: 50 mM HEPES, pH 7.6, 1 mM dithiothreitol, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 0.1% Triton X-100, and 50 μ M ATP. The reaction was stopped by the addition of EDTA to a final concentration of 67 mM (Stephens *et al.* 1997). The reaction mixture was analyzed by immunoprecipitation and a western blot assay. The quantification of the bands was performed by the Gel Analysis V2.02 Software (Clin Science Instruments, Shanghai, China).

Docking analysis

The 3D structure of the IR was obtained from the Protein Database (PDB ID: 1IR3). The mol2 files of ATP (18456332) and adenosine (895113) were obtained from the ZINC database. The binding of the IR tyrosine kinase with ATP and adenosine was studied using the AutoDock 4.2 docking software. The essential hydrogen atoms and the Kollman charges were added. The grid was centered in the active site region, and the grid maps were generated using the Autogrid program. The docking simulations were performed by the Lamarckian Genetic Algorithm. The result of each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 2 500 000 energy evaluations.

Islet preparation and culture conditions

Pancreatic islets were isolated by the collagenase method (Kaiser *et al.* 1988). After 48 h of culture in RPMI 1640 containing antibiotics (100 U/ml penicillin and 100 ng/ml streptomycin) and 10% fetal bovine serum, groups of ten

isolated islets were preincubated for 60 min in the basal medium. The islets then were incubated in the basal medium, either with or without the addition of 10 mM 5'-AMP. The basal medium was the Krebs-Ringer bicarbonate buffer containing 5 mM pyruvate, 5 mM glutamate, 5 mM fumarate, 3.3 mM glucose, and 400 mg/100 ml BSA and was equilibrated with 95% O_2 -5% CO_2 . All of the incubations were carried out at 37 °C for 60 min. At the end of the incubations, aliquots of the incubation media were removed for the insulin assay.

Analysis of adenosine, and nucleotide and adenosine kinase activities

Adenosine and nucleotides were extracted from frozen samples using 0.4 N perchloric acid and analyzed by reverse-phase HPLC (Waters 1525 System; Millipore Corp., Bedford, MA, USA), according to the procedure described previously (Zhang *et al.* 2012). For the isolated pancreatic islets, low concentrations of ATP were assessed using a luminometric method, as described previously (Detimary *et al.* 1996). To assay adenosine kinase (ADK) activities, the liver tissue was homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 3 mM $MgCl_2$, 150 mM KCl, and 0.2 mM phenylmethylsulphonyl fluoride. The homogenate was centrifuged at 50 000 g for 20 min at 4 °C, and the supernatant was stored at -20 °C. The ADK activities were assayed as described previously (Ward *et al.* 1998). Approximately 20 μ g supernatant was incubated for 5 min at 37 °C with a reaction mixture containing 20 mM Tris maleate buffer (pH 5.75) containing 0.5 mM $MgCl_2$, 0.15 mM EHNA, 0.5 mM ATP, 1 mM dithiothreitol and 50 μ M adenosine in a total volume of 80 μ l. The reaction product AMP was measured by HPLC.

RNA extraction and quantitative real-time RT-PCR

RNA extraction and qPCR were performed as described previously (Zhang *et al.* 2012). The following primers were used: *Adk*: 5'-GCCCACCATGGTTCTCATTA-3' (forward), 5'-CCTGCATTGGAAGCAGAAAT-3' (reverse) and *Actb* 5'-GATCATTGCTCCTCCTGAGC-3' (forward), 5'-ACTCCTGCTTGCTGATCCAC-3' (reverse).

Blood glucose and insulin measurements

Blood glucose was determined using a One Touch Blood Glucose Meter (AW063-436-01A; LifeScan, Inc., Milpitas, California, USA) with 3 μ l of whole blood obtained by tail

Figure 1

Regulation of blood glucose by 5'-AMP and insulin. (A) Freely fed mice were treated with a low dose of 5'-AMP (0.5 mmol/kg) by i.p. injection at 0900 h and at 2100 h respectively. Blood glucose levels were measured at 1 h after 5'-AMP injection. (B) Plasma insulin was measured at 0900 h and at 2100 h respectively. (C) The effect of exogenous insulin on 5'-AMP-induced hyperglycemia. Mice were treated intraperitoneally with insulin (Ins)

30 min after injection of 5'-AMP (0.5 mmol/kg). (D) Different responses of blood glucose by i.p. injection of fed mice with various doses of 5'-AMP and insulin. (E) Similar experiments as in (A), but i.p. injection with a high dose of 5'-AMP (1 mmol/kg). Data are expressed as means $\pm$ s.e.m. of four animals. ** $P < 0.01$ compared with saline control. # $P < 0.05$ compared with at 0900 h.

bled. Plasma insulin concentrations were determined by ^{125}I RIA Kits (Linco Research, Inc., St Louis, MO, USA).

Statistical analyses

Data were expressed as mean $\pm$ s.e.m. Statistical analysis was performed by one-way ANOVA, using Tukey's *post hoc* test and Student's *t*-test. Significance was defined as $P < 0.05$.

Results

The blood glucose level was controlled by the ratio of insulin to 5'-AMP

Our previous study had demonstrated that pAMP is an upstream regulator of hyperglycemia in diabetes, and exogenous 5'-AMP increases the intracellular adenosine

level and causes hyperglycemia in WT mice (Zhang *et al.* 2012). The blood glucose and insulin concentration have a daily variation rhythm with a pronounced night peak during feeding time (Ando *et al.* 2005, Takahashi *et al.* 2012). To investigate whether the daily variations of glucose and insulin affect the 5'-AMP-induced hyperglycemia, 5'-AMP (0.5 mmol/kg) was administered to freely fed mice by i.p. injection during the daytime (0900 h) and nighttime (2100 h). At these two time points, similar high glycogen levels were observed in the mice liver (Roesler & Khandelwal 1985). As shown in Fig. 1A, the 5'-AMP injection caused hyperglycemia (over 20 mmol/l blood glucose) at 0900 h, and the same dose of 5'-AMP failed to significantly elevate the blood glucose level at 2100 h. A subsequent measurement of the plasma insulin level indicated that the insulin level at 2100 h was higher than that at 0900 h (Fig. 1B). Figure 1C shows that the

Figure 2

Effects of 5'-AMP on insulin signaling in the liver and skeletal muscle. Fasted mice were treated with insulin and 5'-AMP, and at 10 min after treatment, the liver and the muscle tissue samples were collected. (A and B) Insulin receptor (IR) phosphorylation in the liver and the muscle respectively. Protein extracts were subjected to immunoprecipitation with the anti-phospho-tyrosine antibody. The immunoprecipitates were analyzed by western blot with the anti-IR- β antibody. (C and D) AKT

phosphorylation in the liver and muscle respectively. Protein extracts were analyzed by western blot with the anti-AKT and anti-phospho-AKT (Ser-473) antibodies. The bar graph shows the degree of phosphorylation. The group receiving no stimulation without 5'-AMP was set at 1. Values were expressed as means $\pm$ s.e.m. from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with the group receiving insulin stimulation without 5'-AMP treatment.

exogenous insulin reduced the 5'-AMP-induced hyperglycemia in 30 min after the 5'-AMP injection. Then, the mixed dose of 5'-AMP and insulin was injected into mice, and we found that the absolute level of insulin or pAMP was apparently of less significance than the ratio of these two factors in regulating the blood glucose level (Fig. 1D). A high dose of 5'-AMP (1 mmol/kg) was injected into mice, and the blood glucose level significantly increased at 0900 h and at 2100 h (Fig. 1E). These results suggested that the ratio of insulin to 5'-AMP maintains the glucose homeostasis.

5'-AMP suppressed the insulin-stimulated phosphorylation in the liver and muscle

To clarify the effect of 5'-AMP on the action of insulin, mice that had been fasting were injected with insulin and different doses of 5'-AMP. The results indicated that 5'-AMP caused a dose-dependent inhibition of insulin-stimulated IR tyrosine phosphorylation in the liver and the skeletal muscle tissues (Fig. 2A and B). The maximal tyrosine phosphorylation decreased by 79% ($P < 0.01$) and 58% ($P < 0.01$) in the liver and muscle tissue respectively.

Then, we determined whether the phosphorylation of AKT, which is a downstream target of the IR, changed in response to 5'-AMP. 5'-AMP also caused a dose-dependent inhibition of insulin-stimulated AKT phosphorylation, and the maximal AKT-Ser⁴⁷³ phosphorylation decreased by 46% ($P < 0.05$) and 80% ($P < 0.01$) in the liver and muscle tissue respectively (Fig. 2C and D).

Competition of adenosine and ATP for the ATP-binding site on the IR tyrosine kinase

cAMP plays a key role in the regulation of gluconeogenesis and glycogenolysis (Exton & Park 1968). To investigate whether the 5'-AMP-induced hyperglycemia occurs through the cAMP pathway, 5'-AMP-treated mice were given the adenylate cyclase inhibitor SQ22536. Figure 3A shows that SQ22536 failed to decrease the 5'-AMP-induced hyperglycemia, indicating that 5'-AMP-induced hyperglycemia was not dependent on the cAMP pathway. pAMP-induced hyperglycemia is not directly related to the adenosine receptor pathways, although administration of 5'-AMP causes a significant increase in the hepatic adenosine level (Zhang *et al.* 2012).

Figure 3

Effects of adenosine on insulin receptor autophosphorylation. (A) Freely fed mice were treated with 5'-AMP (0.5 mmol/kg) by i.p. injection at 0900 h. Blood glucose levels were measured at 1 h after 5'-AMP injection. The adenylate cyclase inhibitor SQ22536 (10 mg/kg, s.c.) was administered 30 min before 5'-AMP. HPLC analysis for adenosine levels in the liver (B) and the muscle (C) of fasted mice at 10 min after various doses of 5'-AMP injection. Values are expressed as means $\pm$ s.e.m. of four animals. * $P < 0.05$ and ** $P < 0.01$ compared with saline control. Docking analysis of adenosine and ATP. The docked complexes are ATP and adenosine in (D) and (E)

respectively. Hydrogen bonds were indicated by red dash lines.

(F) Adenosine inhibits IR autophosphorylation *in vitro*. The reaction was performed as described under 'Materials and methods' section. The final results were determined by immunoprecipitation and western blotting. The bar graph shows the degree of phosphorylation. The control was set at 1. Values are expressed as means $\pm$ s.e.m. from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with the group receiving insulin stimulation without adenosine.

Table 1 Results of docking analysis

Compound	Free energy of binding (kcal/mol)	Intermolecular energy	Total internal energy	Torsional free energy	Inhibition constant
ATP	−2.60	−5.88	12.53	3.28	12.46
Adenosine	−3.61	−5.4	−2.46	1.79	2.27

Similarly, at 10 min after the 5'-AMP injection, the levels of adenosine were acutely increased in the liver and muscle tissues of the fasting mice (Fig. 3B and C). We hypothesized that the intracellular hyper-adenosine and ATP were in competition for the ATP-binding site on tyrosine kinase, leading to the reduction in the insulin-stimulated IR autophosphorylation. The docking analysis of adenosine and ATP was performed by Autodock 4.2 and Pymol 1.4. Figure 3D and E show the interaction of the IR tyrosine kinase with ATP and adenosine respectively. ATP was found to interact with Glu-1001, Gln-1004, Asp-1083, Lys-1085, Ser-1086, and Arg-1089, and adenosine was found to interact with Glu-1001, Leu-1002, and Asp-1083. The docking results of the interaction are given in Table 1. The binding energy value of adenosine from Autodock 4.2 was −3.61 kcal/mol, which was lower than that of ATP (−2.6 kcal/mol). The docking results could predict that adenosine was a stronger binding ligand with the IR. The *in vitro* experiments revealed that adenosine caused a dose-dependent inhibition of the insulin-stimulated IR autophosphorylation (Fig. 3F). Adenosine at concentrations of 10 and 50 μ M reduced the autophosphorylation by 22% ($P < 0.05$) and 38% ($P < 0.01$) respectively. These results implied that

the elevated adenosine could dock onto the ATP-binding site of the IR, leading to the inhibition of the ability to autophosphorylate and phosphorylate intracellular substrates.

5'-AMP did not elevate the blood glucose level in the PV-treated mice

The PTPs play a crucial role in the development of insulin resistance in classic metabolic tissues by inhibiting the activation of several molecules in the insulin signaling cascade (Xu *et al.* 2014). PV is an inhibitor of the PTPs and is a potent insulin mimicker (Fantus *et al.* 1989, Heffetz *et al.* 1992), which has been shown to increase the level of the IR phosphorylation (Ruff *et al.* 1997). To investigate whether PV can overcome the 5'-AMP-induced hyperglycemia, mice were given PV by i.p. injection, and after 20 min, mice were treated with different doses of 5'-AMP. Figure 4A shows that neither the high dose (1 mmol/kg) nor the low dose (0.5 mmol/kg) of 5'-AMP caused hyperglycemia in the PV-treated mice. The PV treatment increased the tyrosine phosphorylation of the IR, which was unaltered by the high and low doses of 5'-AMP (Fig. 4B), and the PV-induced phosphorylation of AKT was

Figure 4

5'-AMP failed to cause hyperglycemia in peroxovanadate (PV)-treated mice. (A) At 20 min after administration of PV by i.p. injection, mice were treated with 5'-AMP. Then, blood glucose was measured. Data are expressed as means $\pm$ s.e.m. of four animals. $**P < 0.01$ compared with administration of 5'-AMP. (B) The liver tissue samples were obtained 30 min after i.p. injection of 5'-AMP. Protein extracts were subjected to immunoprecipitation with the anti-phospho-tyrosine antibody. The immunoprecipitates were analyzed by western blot with the anti-IR- β

antibody. (C) Protein extracts were analyzed by western blot with the anti-AKT and anti-phospho-AKT (Ser-473) antibodies. The bar graph shows the degree of phosphorylation. The group without administration of PV and 5'-AMP was set at 1. Values are expressed as means $\pm$ s.e.m. from three independent experiments. (D) HPLC analysis for hepatic adenosine level at 30 min after i.p. injection of 5'-AMP (0.5 mmol/kg). Data are expressed as means $\pm$ s.e.m. of four animals. $**P < 0.01$ compared with saline control. $##P < 0.01$ compared with 5'-AMP treatment.

Figure 5

5'-AMP stimulated insulin secretion and insulin decreased 5'-AMP-induced hyper-adenosine level. The analysis for plasma insulin (A) and ATP/ADP ratio in pancreas (B) at 1 h after i.p. injection of 5'-AMP (0.5 mmol/kg). Data are expressed as means $\pm$ s.e.m. of four animals. * P <0.05 and ** P <0.01 compared with saline control. Pancreatic islets were incubated with or without 10 mM 5'-AMP for 60 min. Insulin secretion (C) and ATP content (D) were assessed. Data are expressed as means $\pm$ s.e.m. of three separate experiments. * P <0.05 compared with saline control. (E) HPLC analysis for

hepatic adenosine level at 20 min after i.p. injection of a mixture of 5'-AMP and insulin (1 U/kg). (F) Mice were treated with a mixture of 5'-AMP and insulin (1 U/kg), and the activity of ADK in the cytosol of livers was assayed. (G) The mRNA expression of *Adk* in the livers. Data are expressed as means $\pm$ s.e.m. of four animals. * P <0.05 and ** P <0.01 compared with saline control. # P <0.05 and ## P <0.01 compared with administration of 5'-AMP (0.2 mmol/kg).

also unchanged (Fig. 4C). The HPLC analysis revealed that PV significantly decreased the cellular adenosine level in the 5'-AMP-treated mice (Fig. 4D).

5'-AMP increased the plasma insulin level and insulin decreased the 5'-AMP-induced hyper-adenosine level

The previous observations revealed that 5'-AMP blocked the insulin signaling and caused hyperglycemia. Then, we tested whether the 5'-AMP injection induced the change in the plasma insulin level. Figure 5A shows that 1 h after the 5'-AMP injection, the plasma insulin level increased by 50% compared with the saline control. The ATP/ADP ratio, which regulates the insulin secretion, also increased in the pancreatic tissues (Fig. 5B). Similarly, while the isolated islets were exposed for 60 min to 5'-AMP, the insulin release increased compared with the saline control (Fig. 5C). The ATP level in the islets also increased (Fig. 5D). Then, we investigated the effect of insulin on the intracellular adenosine concentration. For the high dosage of 0.5 mmol/kg 5'-AMP, the insulin injection only resulted in a decreasing tendency, whereas for mice treated with 0.2 mmol/kg 5'-AMP, the insulin injection significantly decreased the 5'-AMP-induced hyper-adenosine level in the liver (Fig. 5E), which was associated with an increase in the enzymatic activities of ADK (Fig. 5F). Insulin failed to significantly enhance the

expression of the *Adk* mRNA with a high or low dosage of the 5'-AMP treatment (Fig. 5G).

Discussion

Glucose homeostasis is under a complex and only partially understood neuro-hormonal regulation. Among the hormonal factors, insulin is the primary hormone that lowers the blood glucose by inhibiting glucose production and stimulating glucose utilization in insulin-sensitive tissues, whereas glucagon and glucocorticoids are the major hormones that can increase the plasma glucose concentrations (DeFronzo 1988, Gerich 1993). Endothelial cell damage of the veins caused by FFA elevated the pAMP levels in the *db/db* diabetic mice, and mice treated with 5'-AMP showed induced hyperglycemia and displayed patterns similar to the *db/db* mice in several glucose metabolic gene expressions and physiological responses (Zhang *et al.* 2012). In this study, we demonstrated that 5'-AMP, as a counter-regulatory factor of insulin, impairs the insulin signaling and elevates the blood glucose level. The ratio of 5'-AMP to insulin controls the blood glucose level. The plasma insulin concentration had a daily variation and a peak at nighttime (Ando *et al.* 2005), which resulted in different changes in the blood glucose after the 5'-AMP treatment in the daytime and in the nighttime. The finding that 5'-AMP impaired the insulin

action implied that 5'-AMP was a primary factor causing insulin resistance in type 2 diabetes.

Extracellular 5'-AMP (including pAMP) cannot be directly transferred into intracellular 5'-AMP. At the early stage of the 5'-AMP injection, the intracellular 5'-AMP failed to increase, but the intracellular adenosine sharply increased, resulting in an increase in the intracellular ATP level (Weisman *et al.* 1988) and decreasing the ratio of 5'-AMP/ATP. A decrease in the cellular ratio of 5'-AMP/ATP should lead to a decrease in the AMPK activity and glucose utilization (Hardie *et al.* 1998). Hyper-adenosine also enhances the activity of glucose-6-phosphatase, resulting in an increased glycogenolysis and endogenous glucose production (Zhang *et al.* 2012). Thus, glycogenolysis plays a key role in 5'-AMP-induced hyperglycemia. It is well known that the glycogen level displays a daily variation in the mouse liver with a decrease in daytime (Roesler & Khandelwal 1985). A different injection time with 5'-AMP will cause a different increase in blood glucose, which is dependent on hepatic glycogen level in these time points (Zhang *et al.* 2006, 2012). In diabetic mice, pAMP and hepatic adenosine are maintained constantly at a high level; 5'-AMP injection in normal mice only results in sharply increases in pAMP and hepatic adenosine at the first stage and causes a temporary elevation in blood glucose levels. At the last stage of the 5'-AMP injection, the increased adenosine level is primarily phosphorylated to 5'-AMP by ADK (Arch & Newsholme 1978); increased intracellular 5'-AMP enhances activities of AMPK and phosphofructokinase and the inhibition of the activity of fructose-1,6-bisphosphatase, resulting in an increased glucose utilization and reduced gluconeogenesis (Zhang *et al.* 2006). The proposed possible scheme of this regulatory event is shown in Fig. 6.

5'-AMP has no influence on the affinity of insulin binding to its receptor (Zhang *et al.* 2012). Our results indicate that 5'-AMP causes a dose-dependent inhibition of the insulin-stimulated IR autophosphorylation in the liver and muscle tissues, inhibiting the activity of the receptor tyrosine kinase and suppressing the phosphorylation of downstream proteins. The docking analysis revealed that adenosine is a stronger binding ligand with the IR compared with ATP. In addition, 5'-AMP attenuated the insulin-stimulated AKT phosphorylation in the liver and muscle tissues. AKT, a serine/threonine kinase, is a downstream target of the IR, and it has been implicated as a key signaling protein for several of the actions of insulin, including activation of glycogen synthesis, protein synthesis, and GLUT4 translocation to the cell surface, thereby increasing glucose transport (Whiteman *et al.*

Figure 6

Schematic of the effects of 5'-AMP on blood glucose over time. At the early stage of 5'-AMP injection, the intracellular adenosine sharply increased, which impaired the action of insulin, reduced the activity of AMPK, and enhanced the activity of G6Pase, resulting in an increase in blood glucose. At the last stage of the 5'-AMP injection, the increased intracellular 5'-AMP enhanced the activities of AMPK and phosphofructokinase and the inhibition of the activity of fructose-1,6-bisphosphatase, leading to decreased blood glucose levels from high to baseline or lower. IR, insulin receptor; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase. Solid line, positive regulation; Dashed line, negative regulation.

2002, Guo 2014). The decreased AKT phosphorylation is due to the reduced IR autophosphorylation and might be a result of the inhibitory activities of other upstream kinases including AKT by adenosine binding to the ATP-binding site of these kinases. In diabetic *db/db* mice, insulin-stimulated AKT phosphorylation in skeletal muscle decreased compared with WT mice (Shao *et al.* 2000). The reduced AKT activity may contribute to the cellular mechanism for the decreased GLUT4 translocation, the increased expression of the gluconeogenic genes, and the insulin resistance (Shao *et al.* 2000, Whiteman *et al.* 2002, Puigserver *et al.* 2003, Guo 2014). PV, an inhibitor of PTPs and a potent insulin mimicker (Fantus *et al.* 1989, Heffetz *et al.* 1992), significantly reversed the 5'-AMP-induced decrease in IR and the downstream phosphorylation of the kinases. Unexpectedly, PV significantly inhibited the 5'-AMP-induced hyper-adenosine in the liver. All of this evidence revealed that cellular adenosine plays a key role in the 5'-AMP-induced hyperglycemia.

As a second messenger, cAMP stimulates gluconeogenesis and glycogenolysis in the liver (Exton & Park 1968). We found that 5'-AMP-induced hyperglycemia was not dependent on the cAMP pathway. The hyper-adenosine level is the primary factor. Our results revealed that adenosine was a stronger binding ligand with the IR compared with ATP, inhibiting the insulin-stimulated receptor tyrosine phosphorylation and impairing the action of insulin. In the early stage of type 2 diabetes, a high level of insulin circulates in the blood because the

pancreas can still produce the hormone (Zhou *et al.* 1999). 5'-AMP injection not only impairs the insulin action but also stimulates the plasma insulin level, which is similar to insulin resistance. In pancreatic β cells, ATP plays critical roles in regulating the insulin secretion. An increase in cytosolic ATP or the ATP/ADP ratio induces the closure of the ATP-sensitive potassium channel, resulting in depolarization of the plasma membrane to allow for Ca^{2+} influx, which triggers insulin secretion from cells (Detimary *et al.* 1998). 5'-AMP injection resulted in an elevation in the ATP level in the isolated islets and the pancreas, which could contribute to the stimulation of the insulin production.

In conclusion, 5'-AMP increases the plasma insulin level and impairs the insulin signaling via intracellular hyper-adenosine, leading to a reduction in the insulin responsiveness. Insulin decreases the level of 5'-AMP-induced hyper-adenosine in the liver. Blood glucose homeostasis is reciprocally regulated by pAMP and insulin. The present data could be useful for understanding the crucial role of pAMP in the insulin resistance of type 2 diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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