Glucose decreases extracellular adenosine levels in isolated mouse and rat pancreatic islets

Gary K. Yang,1 Paul E. Squires,2 Faming Tian,1,4 Timothy J. Kieffer,1,4 Yin Nam Kwok1 and Nicholas Dale2,*

1Department of Cellular and Physiological Sciences; University of British Columbia; Vancouver, BC Canada; 2School of Life Sciences; University of Warwick; Coventry, West Midlands, UK; 3Sarissa Biomedical Ltd.; Coventry, West Midlands, UK; 4Department of Surgery; University of British Columbia; Vancouver, BC Canada

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Introduction

The pancreatic islets of Langerhans are responsible for the regulated release of the endocrine hormones insulin and glucagon that participate in the control of glucose homeostasis. Abnormal regulation of these hormones can result in glucose intolerance and lead to the development of diabetes. Numerous efforts have been made to better understand the physiological regulators of insulin and glucagon secretion. One of these regulators is the purine nucleoside, adenosine. Though exogenous application of adenosine has been demonstrated to stimulate glucagon release and inhibit insulin release, the physiological significance of this pathway has been unclear. We used a novel 7 μm enzyme-coated electrode biosensor to measure adenosine levels in isolated rodent islets. In the mouse islets, basal adenosine levels in the presence of 3 mM glucose were estimated to be 5.7 ± 0.6 μM. As glucose was increased, extracellular adenosine diminished. A 10-fold increase of extracellular KCl increased adenosine levels to 16.4 ± 2.0 μM. This release required extracellular Ca2+ suggesting that it occurred via an exocytosis-dependent mechanism. We also found that while rat islets were able to convert exogenous ATP into adenosine, mouse islets were unable to do this. Our study demonstrates for the first time the basal levels of adenosine and its inverse relationship to extracellular glucose in pancreatic islets.

Adenosine signaling is emerging as a candidate regulator of glucose metabolism and systemic energy homeostasis. The two hormones that have received the most attention in both basic and clinical research fields are insulin from β-cells and glucagon from α-cells, which mediate opposing actions on glucose metabolism. While insulin is responsible for inhibiting hepatic gluconeogenesis and promoting glucose uptake into peripheral tissues, thereby decreasing blood glucose levels; glucagon stimulates gluconeogenesis and glycogenolysis, thereby increasing blood glucose levels. The regulated release of insulin and glucagon is, therefore, responsible for the tight control of blood glucose levels in healthy individuals. Insufficient levels or activity of insulin, and abnormal elevations of glucagon have been associated with the development of impaired glucose tolerance leading to diabetes mellitus.1 Therefore, understanding the physiological regulation of these two hormones and the changes of these regulatory mechanisms in pathological states may lead to further therapeutic options.

Adenosine signaling is emerging as a candidate regulator of insulin and glucagon release. Adenosine is an endogenous purine nucleoside that is present in all cell types of the body. Over the last few decades, the direct receptor-dependent signaling pathways of adenosine have gained attention for their therapeutic potential. Adenosine carries out its physiological effects by activating surface adenosine receptors, of which there are four subtypes identified: A1, A2A, A2B and A3.2 These adenosine receptors have been found to be ubiquitously distributed throughout the body in a non-uniform manner and numerous physiological functions of adenosine have been characterized.3 A previous study identified adenosine A1 receptors on β- and α-cells as well as adenosine A2A receptors on α-cells, suggesting a potential direct adenosine signaling pathway on the release of these hormones.4 Studies have shown that adenosine and A1 receptor agonists inhibit insulin release in the perfused rat pancreas5 and in the β-cell tumor cell line, INS-1.6 Adenosine and its analogs have also been demonstrated to stimulate glucagon release in the perfused rat pancreas in a concentration-dependent manner6 and to potentiate arginine-induced glucagon secretion.7 Although the pharmacological effects of adenosine on inhibiting insulin secretion and potentiating glucagon secretion have been postulated, evidence for the endogenous release of adenosine in the pancreas is lacking.

Indirect evidence for the production of adenosine by islet cells has come from examination of the release of ATP. ATP is present...
in insulin-containing granules of β-cells10–13 and has been shown to be released following stimulation prior to the release of insulin.14 ATP can also be released in a regulated manner from sympathetic and parasympathetic nerves as well as intrinsic nerves within pancreatic islets.15–18 The metabolism of released ATP could potentially lead to changes in extracellular adenosine levels in islets. However, a recent study suggested that the enzyme required for the last step of converting ATP into adenosine, ecto-5' nucleotidase, may not be present in mouse and human islets, although this enzyme is present in rat islets.19 Therefore, the notion of ATP being the source of adenosine in pancreatic islets may be species-specific. In addition, it remains unclear how adenosine levels may vary during different metabolic conditions.

Under physiological conditions, the half-life of extracellular adenosine has been estimated to be less than 10 sec20 but accurate real-time measurements characterizing the changes of adenosine level have proved to be challenging. Enzyme-coated electrode biosensors have been developed to enable the real-time measurement of adenosine levels directly in tissues.21,22 In this study, similar biosensors were used to determine the levels of adenosine in mouse and rat islets. Changes in adenosine levels under different glucose conditions were also assessed. In addition, the mechanisms involved in the release of adenosine were also examined.

**Results**

Effect of exogenous glucose on extracellular adenosine levels. To determine the adenosine concentration in isolated mouse islets, the adenosine and null biosensors (Fig. 1, see Materials and Methods) were placed in the proximity of a pancreatic islet. Current changes were not detected on either the adenosine biosensor or the null sensor. To increase the contact surface area between the biosensor enzymes and the pancreatic islets, the electrodes were inserted through the center of the islets. In order to fully cover the enzyme-coated regions of the electrodes with islets, multiple islets were penetrated in sequence (Fig. 2A). Levels of adenosine were extrapolated by comparing the difference in the currents measured from the adenosine and the null biosensors against a standard curve, which had the baseline measured after removing the islets. The rapid increases in adenosine levels following each penetration returned to a steady-state within 10 min. Although basal adenosine levels during perfusion of 3 mM glucose remained relatively steady for each islet preparation, variations between different preparations and islets from different animals were apparent. Furthermore, the number of islets that were effectively attached to the sensors in each experiment also varied. Basal adenosine concentration from different islet preparations ranged from 1.2–11.6 μM with an average of 5.7 ± 0.6 μM (n = 27). To account for this variation, each set of islet preparations served as its own control in all experimental conditions.

To determine the relationship between extracellular glucose concentration and adenosine levels in pancreatic islets, glucose concentrations between 0–25 mM were tested. A decrease in glucose concentration from 3–0 mM caused an increase in adenosine levels (Fig. 2B). Conversely, an increase in glucose concentration from 3 mM to 5–25 mM caused a decrease in adenosine levels (Fig. 2C and D). Furthermore, glucose concentrations above 8 mM did not seem to cause any further decrease in adenosine levels. These results suggest that glucose decreases adenosine levels in mouse islets with maximum inhibition achieved at glucose concentrations ≥ 8 mM. This inverse glucose-adenosine relationship was well fitted by the Hill equation with a dissociation constant of 4.6 mM and a Hill coefficient of 3 (Fig. 2D).

Mechanisms involved in the release of adenosine in the mouse islets. To determine whether adenosine is released from islet cells via an exocytosis-dependent mechanism or via nucleoside transporters, we investigated the effect of KCl-induced membrane depolarization of the islet cells. In the presence of 30 mM KCl, adenosine concentration increased by 3-fold (Fig. 3A and C). In addition, this effect of KCl was only apparent in the presence of
Ca\(^{2+}\). In the absence of extracellular Ca\(^{2+}\), basal adenosine levels were lower and did not respond to exogenous KCl (Fig. 3B and C). Since Ca\(^{2+}\) influx is required for exocytosis to occur, the lower adenosine concentrations and the lack of an effect of KCl in the absence of Ca\(^{2+}\) suggest an exocytosis-dependent source of extracellular adenosine in the mouse islets. To determine whether adenosine is also released through nucleoside transporters, the effects of the nucleoside transporter blockers, NTBI and dipyridamole, were investigated. In the presence of NTBI (50 \(\mu\)M) alone or in combination with dipyridamole (10 \(\mu\)M), adenosine concentrations were not significantly different from control levels (Fig. 3). These results suggest that the nucleoside transporters are unlikely to be involved in the generation of basal adenosine levels.

To determine whether adenosine is released from the islets as adenosine or as a consequence of ATP metabolism, we used an ATP biosensor. The ATP biosensor did not detect any basal ATP levels and was not responsive to exogenous KCl (Fig. 4A). We added exogenous ATP to determine whether it could be rapidly broken down into adenosine in the extracellular space. In the presence of ATP, adenosine levels did not significantly change (Fig. 4A). To test the possibility that ATP could be packaged into exocytotic granules and converted to adenosine by granular nucleotidases, exocytosis was induced by KCl followed by infusion of ATP. In the presence of KCl, extracellular adenosine levels increased; however, exogenous infusion of ATP did not induce a further increase in adenosine concentration (Fig. 4A and B). These studies suggest that extracellular adenosine in the islets is unlikely to arise from the breakdown of ATP.

Regulation of adenosine levels in the rat islet. The observation that the administration of ATP to the isolated mouse islets did not increase extracellular adenosine levels (Fig. 4B) is in agreement with the previous observation that the extracellular enzyme responsible for converting AMP into adenosine, ecto-5’-nucleotidase, was not identified in the mouse islets.\(^{19}\) However, in the same study, the authors did find ecto-5’-nucleotidase immunoreactivity in rat islets. To determine whether rat islets are thus capable of converting ATP to adenosine, exogenous ATP was administered. In the presence of 100 \(\mu\)M ATP, adenosine levels increased and this occurred also in the absence of extracellular Ca\(^{2+}\) (Fig. 5A and B). Furthermore, the nucleo-
tidase inhibitors, POM-1 (inhibitor of NTPDases) and GMP (inhibitor of ecto-5'-nucleotidase), inhibited the increased adenosine levels observed with exogenous ATP (Fig. 5B). The non-metabolizable analog of ATP, α, β-methylene ADP, which is also an inhibitor of the ecto-5'-nucleotidase, did not cause an increase in adenosine levels (Fig. 5B).

To determine whether or not adenosine levels in rat islets are also regulated by glucose in a Ca^{2+}-dependent mechanism, different conditions were tested. Adenosine levels in the rat islets were higher in the presence of 3 mM glucose compared with that in the presence of 16.7 mM glucose (Fig. 5C). Furthermore, exogenous infusion of 30 mM KCl caused an increase in adenosine levels. As in mouse islets (Fig. 3C), the KCl-induced increase in adenosine levels only occurred in the presence of Ca^{2+} (Fig. 5C).

Islet cell type responsible for the release of adenosine. The two main islet cell types in the endocrine pancreas are the insulin-releasing β-cells and the glucagon-releasing α-cells. To determine whether or not adenosine was released from these two cell types, we used l-arginine and forskolin. In the presence of low extracellular glucose, both l-arginine and forskolin stimulate glucagon release while in the presence of high extracellular glucose they stimulate insulin release. At 3 mM glucose, neither l-arginine nor forskolin affected adenosine release (Fig. 6). Although adenosine levels were lower when 16.7 mM glucose was perfused, l-arginine and forskolin did not affect adenosine release compared with glucose alone controls (Fig. 6).

Discussion

In this study, basal levels of adenosine in isolated mouse islets were determined to be in the micromolar range. This is highly relevant as this concentration of adenosine will activate adenosine receptors and has been previously shown to stimulate glucagon release and inhibit insulin release. Furthermore, a previous study has suggested that adenosine receptor activation in the islets may delay or prevent the onset of autoimmune type 1 diabetes due to immunomodulation. Our data therefore suggest that adenosine is not only a physiologically relevant and significant regulator of the release of pancreatic hormones but may also be a potential candidate in treating diabetes.

We have also demonstrated for the first time that adenosine levels are inversely correlated with extracellular glucose concentrations in mouse islets. This is consistent with the proposed role of adenosine as an insulin inhibitor and a glucagon secretagogue. Thus
under low glucose conditions, endogenous adenosine levels are sufficiently high to inhibit insulin release and stimulate glucagon release. Conversely, under elevated glucose conditions, adenosine levels decrease along with glucagon release while insulin release is augmented. Therefore, our data suggest that adenosine may act as a paracrine or autocrine signal in the pancreatic islets and thereby contribute to the regulation of hormone secretion.

Adenosine in a tissue typically arises from cellular release via nucleoside transporters or from the breakdown of adenine nucleotides by ectonucleotidases.26 The islets of Langerhans are composed mainly of endocrine cells that secrete various vesicle-stored hormones via exocytosis. In addition, purine nucleotides such as ATP have been postulated to be released in conjunction with the stored hormones.10-14 Therefore, it is possible that the extracellular adenosine in the islets could be derived from an exocytotic source. This was indeed demonstrated in the present study whereby depolarizing the islet cells with a high concentration of KCl induced an increase in adenosine levels and this only occurred in the presence of extracellular Ca^{2+}, which is required for exocytosis to occur. In addition, the lack of an effect of the ATP analog 2',3'-methylene ADP (resistant to hydrolysis) did not affect adenosine levels. Our data, therefore, suggest that rat islets have ectonucleotidases necessary to convert ATP to adenosine and is in agreement with the previous immunohistological study demonstrating that rat islets contain ecto-5' nucleotidase immunoreactivity.26

Glucagon secretion is higher under low glucose conditions and is inhibited by higher glucose conditions. Thus, the inversely-correlated levels of adenosine and glucose are consistent with the hypothesis that the glucagon-releasing β-cells may be the source of adenosine release. However, results of the present study do not support this hypothesis. In the presence of low extracellular glucose and in the presence of the secretagogues, t-arginine and forskolin that would stimulate the β-cells, adenosine levels were not significantly altered. Both t-arginine and forskolin while being secretagogues of glucagon under low extracellular glucose stimulate insulin release under high extracellular glucose.29,30

However, under both glucose conditions, neither secretagogue, at concentrations that have been previously demonstrated to affect insulin and glucagon release,29,30 had a significant effect on adenosine levels compared with basal levels. Despite the variations observed within a treatment group, we did not observe any significant changes in adenosine concentration following administration of the secretagogues that would have been expected if adenosine release was in conjunction with the islet hormones. The simplest interpretation of our results is that perhaps neither t-arginine nor forskolin would stimulate the β-cells to release adenosine, and that it is not co-released with either insulin or glucagon. However, these cell types could be cellular sources of adenosine were packaged in a different subset of secretory granules that were regulated in a different way from glucagon- or insulin-containing granules. GABA has been postulated to reside in a different population of synaptic-like secretory granules from insulin in the β-cells and be released by exocytosis under different conditions from the insulin granules.21-23 Therefore, the cellular source of adenosine release is at present unclear.

Although numerous studies have demonstrated that ATP is co-released with insulin from β-cells,24-26 the present studies using an ATP biosensor did not detect any basal levels of ATP or any
changes in ATP following glucose or KCl stimulation. It is possible that ATP is released in a site-specific manner and that the breakdown of ATP into ADP or AMP occurred too rapidly for the biosensor to detect these changes. Furthermore, in the rat islets that had the necessary enzymes to break ATP into adenosine, ATP levels were also not detectable. It is, therefore, unclear at this point as to why the ATP biosensor was not able to measure ATP in the isolated islets and more studies are thus warranted.

In conclusion, we have used novel 7 μm carbon fiber enzyme-coated electrode biosensors to demonstrate for the first time that adenosine levels in the mouse and rat islets are inversely correlated with glucose levels. Furthermore, we determined that adenosine is released through an exocytosis-dependent mechanism. Our study also demonstrated the first functional evidence that while mouse islets cannot breakdown extracellular ATP into adenosine, rat islets can. The exact physiological consequences of this difference remains to be elucidated. Since human islets have also been suggested to not possess ecto-5’ nucleotidase, mice may be a better model for the study of adenosine in islet physiology. Future studies should focus on examining the specific cell types in islets that are responsible for the release of adenosine. The biosensor technology we developed may be applied to determine real-time changes in adenosine levels in other small tissues or regions.

Materials and Methods

Isolation and culture of pancreatic islets. Isolation procedures of mouse pancreatic islets were essentially as previously described. Sprague-Dawley rats (16–22 d old) and B6 CB F1 mice (5–6 weeks old) were sacrificed by cervical dislocation in accordance with the UK Animals (Scientific Procedures) Act 1986. The abdominal cavity was opened up to expose the bile duct. The common bile duct at the junction with the duodenum was carefully clamped off with a hemostat to prevent flow into the duodenum. Collagenase type XI (1000 U/ml; Sigma-Aldrich) was prepared in modified Hank’s buffered salt solution (HBSS) composed of (mM): 137 NaCl, 5.4 KCl, 4.3 NaH2PO4, 1.4 KH2PO4, 10 HEPES, 1 MgCl2, and 5 glucose. Collagenase solution was injected into the bile duct away from the liver and toward the pancreas until the entire pancreas was expanded (approximately 1 ml). The pancreas was then dissected away from the rest of the animal and placed into a 15 ml tube with 2 ml of collagenase solution and kept in a water bath (37°C) for 14 min. The pancreas was then dissected away from the liver and placed into a 15 ml tube with 2 ml of HBSS containing 10 mM KH2PO4, 10 HEPES, 1 MgCl2, and 5 glucose. Collagenase solution was injected into the bile duct away from the liver and toward the pancreas until the entire pancreas was expanded (approximately 1 ml). The pancreas was then dissected away from the rest of the animal and placed into a 15 ml tube with 2 ml of collagenase solution and kept in a water bath (37°C) for 14 min. Immediately following digestion, 5 ml of HBSS containing 1 mM CaCl2 was added and the tube was kept on ice. The contents were then poured onto a culture dish and islets were picked out and placed in clean HBSS containing CaCl2. Islets in groups of 15–20 were cultured in a 35 by 10 mm tissue culture dish with a 22 by 22 mm glass cover slide and 2 ml of Roswell Park Memorial Institute (RFMI) 1640 media supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The culture dishes were incubated at 37°C with 5% CO2 overnight to allow attachment of islets to the cover slides.

Biosensors. The generation of the carbon fiber biosensors used in this study is similar to the previously described procedures for the generation of platinum biosensors. The standard platinum-based biosensors have a diameter of 25 μm and penetrating the isolated endocrine islets proved to be difficult with these biosensors. Therefore, novel carbon fiber biosensors with a 7 μm diameter were used. Adenosine biosensors were obtained from Sarissa Biomedical Ltd. The biosensors consisted of enzymes entrapped in the polymer at the tip of the carbon fiber electrode. The enzymes on the biosensors could break down adenosine around the electrode into H2O2, which was further oxidized into a measurable current (Fig. IC). Currents in the electrodes were measured by a potentiostat (Duostat ME200+; Sarissa Biomedical Ltd.) and recorded on an attached computer. The null sensor did not contain enzymes and served as a negative control for the biosensors. Both current recordings from the adenosine and the null biosensors were taken and the difference between the two readings was used to generate the standard curve (Fig. I). The standard curve demonstrated that the adenosine biosensors can most accurately determine adenosine concentrations between 1–20 μM. None of the drugs or nutrients used in this study interfered with the adenosine measurements by the biosensors directly. ATP biosensors used were also calibrated in a similar manner, which has been previously described.44

Perfusion of islets. The islets were perfused with modified Krebs solution composed of (mM): 117 NaCl, 3 KCl, 26 NaHCO3, 1.25 NaH2PO4, 1 MgSO4, 1 CaCl2, 3 glucose and 22 sucrose. For solutions containing 30 mM KCl, equimolar amounts of sucrose were removed. For different glucose concentrations and for 10 mM l-arginine solutions, equimolar amounts of sucrose were removed. For Ca2+-free solutions, equal molar amounts of MgCl2 replaced CaCl2 and 1 mM EGTA was added to chelate residual Ca2+ or Ca2+ released from the tissue. For all solutions used with the ATP biosensor, 2 mM glycerol was included as a co-substrate for ATP detection. Steady infusion was performed at a rate of 2 ml/min by a peristaltic pump with a vacuum suction to maintain constant fluid level. All solutions were heated to 37°C prior to entry into the perfusion chamber. Adenosine and null sensors were gently lowered into the perfusion chamber care was taken to ensure that the tips of the electrodes remained in the bath at all times, thus allowing the signal to reach a steady-state. A standard solution containing 10 mM adenosine was used before the experiment to calibrate the standard curve and also following the experiment to ensure biosensor health.

For experiments, glass cover slides with islets attached were placed into a perfusion chamber. With the aid of a stereomicroscope adenosine biosensors were gently lowered to penetrate four to five islets in a row to fully cover the enzyme portion of the electrode. Null sensors were also lowered to penetrate another subset of islets present on the coverslip. Following penetration with the adenosine biosensor (but not the null sensor), a sharp increase in the current recording was noted after which it declined to a steady-state level. This acute rise in adenosine levels was likely due to cellular damage during insertion of biosensor that caused subsequent release of intracellular purines. To ensure a steady-state was reached in all the islets following electrode penetration, the islets were continuously perfused with the basal solution containing 3 mM glucose for 20 min prior to experiments.
Repeated measures ANOVA was also used to analyze the glucose-adenosine relationship in Figure 2D followed by Dunn’s multiple comparison test to compare all glucose treatments to the control at 3 mM glucose. Statistical significance was considered when p ≤ 0.05.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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