Inosine-Stimulated Insulin Release and Metabolism of Inosine in Isolated Mouse Pancreatic Islets

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Inosine is a potent primary stimulus of insulin secretion from isolated mouse islets. The inosine-induced insulin secretion was totally depressed during starvation, but was completely restored by the addition of 5 mM-caffeine to the medium and partially restored by the addition of 5 mM-glucose. Mannoheptulose (3 mg/ml) potentiated the effect of 10 mM-inosine in islets from fed mice. The mechanism of the stimulatory effect of inosine was further investigated, and it was demonstrated that pancreatic islets contain a nucleoside phosphorylase capable of converting inosine into hypoxanthine and ribose 1-phosphate. Inosine at 10 mM concentration increased the lactate production and the content of ATP, glucose 6-phosphate (fructose 1,6-diphosphate + triose phosphates) and cyclic AMP in islets from fed mice. In islets from starved mice inosine-induced lactate production was decreased and no change in the concentration of cyclic AMP could be demonstrated, whereas the concentration of ATP and glucose 6-phosphate rose. Inosine (10 mM) induced a higher concentration of (fructose 1,6-diphosphate + triose phosphates) in islets from starved mice than in islets from fed mice suggesting that in starvation the activities of glyceraldehyde 3-phosphate dehydrogenase or other enzymes below this step in glycolysis are decreased. Formation of glucose from inosine was negligible. Inosine had no direct effect on adenylate cyclase activity in islet homogenates. The observed changes in insulin secretion and islet metabolism mimic what is seen when glucose and glyceraldehyde stimulate insulin secretion, and as neither ribose nor hypoxanthine-stimulated insulin release, the results are interpreted as supporting the substrate-site hypothesis for glucose-induced insulin secretion according to which glucose has to be metabolized in the β -cells before secretion is initiated.

It is assumed that glucose initiates insulin secretion by interaction with a cellular receptor, but the precise nature of this receptor has not yet been clearly defined. It has been suggested that excitation of the secretory process by glucose may involve metabolism of the sugar, the 'substrate-site hypothesis' (Grodsky *et al.*, 1963; Coore & Randle, 1964) or that secretion is triggered by a direct binding of the glucose molecule to the receptor (Randle *et al.*, 1968; Cerasi & Luft, 1970). A combination of these mechanisms may also be a feasible model for glucose-induced secretion (Ashcroft *et al.*, 1973*b*). Studies with glyceraldehyde (Ashcroft *et al.*, 1973*b*; Hellman *et al.*, 1974) have brought strong evidence in favour of the substrate-site hypothesis and have led to the assumption that insulin release is initiated by a glucose metabolite at or below the triose phosphate level.

In the present work we report observations on inosine-stimulated insulin release which are taken as further support for the substrate-site hypothesis. Inosine is a potent primary stimulus of insulin secretion in isolated mouse islets. The mechanism behind

the stimulation was investigated, and it is concluded that inosine acts as an insulin secretagogue by being first split to hypoxanthine and ribose 1-phosphate through the action of a nucleoside phosphorylase. The ribose part of the inosine molecule is then further metabolized through the pentose phosphate cycle with the concomitant formation of glyceraldehyde 3-phosphate, lactate, glucose 6-phosphate and other intermediates in the glycolytic pathway, one of which may 'trigger' insulin secretion.

Experimental

Materials

¹²⁵I-labelled pig insulin, mouse insulin and insulin antibody were generous gifts from Dr. J. Schlichtkrull, Novo Research Institute, Copenhagen, Denmark. Firefly luciferase and luciferase from *Achromobacter fischerii* were from Sigma Chemical Co., St. Louis, MO, U.S.A. Human serum albumin was from Behringwerke A.G., Marburg-Lahn, Germany. Cyclic [8-³H]AMP (27.5 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Calf thymus DNA was from BDH Chemicals Ltd., Poole, Dorset, U.K. Enzymes, including collagenase, and coenzymes were from Boehringer, Mannheim, Germany. Other reagents used were of the purest grade obtainable.

Methods

Preparation of islets. Pancreas from male albino mice (Theillers original strain; Tuck and Son, Raleigh, Essex, U.K.) were used. For each experiment 12–20 mice of the same age and weight (20–22 g) were selected and divided into two groups. One group was fed on a standard laboratory diet *ad libitum* for 24 h and the other was starved for 48 h (with free access to drinking water) before being killed by decapitation. Starvation was carried out in wire-bottomed metabolic cages to minimize coprophagy. Islets were separated by a collagenase method and harvested with a loop as described by Coll-Garcia & Gill (1969).

Insulin release. Batches of five islets were incubated (with shaking) in 0.3 ml of Krebs bicarbonate medium (Krebs & Henseleit, 1932) containing human serum albumin (2 mg/ml) and other additions as stated. The tubes were incubated at 37°C for 2 h, gently centrifuged and a sample (0.1 ml) of the medium was removed, diluted with a 0.04 M-sodium phosphate buffer, pH 7.4, containing human serum albumin (10 mg/ml) and stored at –20°C until assay by radioimmunoassay (Heding, 1966) with mouse insulin as standard.

Nucleoside phosphorylase assay. Some 200 islets were collected in 50 µl of a 0.1 M-phosphate buffer, pH 7.4. A homogenate was made by sonication, and the nucleoside phosphorylase activity was measured in 1 ml of the same buffer supplemented with inosine (0.22 mM) and xanthine oxidase (0.012 unit/ml). The E_{293} of the uric acid formed in this reaction was measured; the enzyme activity was linear for at least 1 h.

Lactate production. Some 100 islets were collected in 100 µl of the Krebs bicarbonate buffer with no further addition, or supplemented with 10 mM-inosine, and incubated for 2 h at 37°C. HCl (0.075 M; 150 µl) was added and the tubes were heated at 60°C for 60 min. After sonication and centrifugation the lactate in the supernatant was assayed as described by Ashcroft *et al.* (1970).

ATP assay. ATP was measured by means of firefly luciferase as described in detail by Hedekov & Capito (1974).

Fructose diphosphate assay. The content of fructose 1,6-diphosphate was determined as described by Ashcroft *et al.* (1973a). Fructose 1,6-diphosphate was converted into 3-phosphoglyceric acid and the NADH produced was measured in a liquid-scintillation spectrometer after the addition of bacterial luciferase.

The assay measures the sum of fructose 1,6-diphosphate and triose phosphates.

Glucose 6-phosphate assay. Glucose 6-phosphate was converted into 6-phosphogluconate and the NADPH produced was measured by means of bacterial luciferase as described by Ashcroft *et al.* (1973a).

Cyclic AMP assay. Cyclic AMP was determined by a protein-binding radioassay as described by Capito & Hedekov (1974).

DNA content. DNA content of samples of the homogenate was assayed by the methods of Kissane & Robbins (1958) with calf thymus DNA as standard. The fluorimeter was a thermostatically controlled Aminco-Bowman spectrofluorimeter.

Glucose assay. Some 15 islets were incubated in 15 µl of gassed ($O_2 + CO_2$, 95:5) Krebs-Henseleit (1932) buffer, pH 7.4, or in the same buffer containing 10 mM-inosine. The glucose concentration in the incubation medium (where all the glucose was found) was determined as the NADPH formed after the addition of hexokinase, glucose 6-phosphate dehydrogenase and NADP⁺ by the aid of bacterial luciferase as described by Ashcroft *et al.* (1973a).

Adenylate cyclase assay. Some 100–200 islets were collected in 150 µl of 25 mM-Tris buffer, pH 7.6, containing 5 mM-MgCl₂ and 1 mM-EDTA. A homogenate was prepared by sonication. A sample of the homogenate was added to the reaction mixture, which consisted of 25 mM-Tris buffer, pH 7.6, with 1 mM-EDTA, 0.15% human serum albumin, 5 mM-MgCl₂, 1.5 mM-3-isobutyl-1-methylxanthine, and 3.0 mM-ATP and incubated for 30 min at 30°C. The total incubation volume was 40 µl. The reaction was stopped by the addition of 8 µl of 2.5 M-H₂SO₄. The non-cyclic nucleotides were precipitated by the addition of 16 µl of 1 M-barium acetate and the precipitate was removed by centrifugation. The pH of the supernatant was adjusted to 5.5 with 0.4 M-trisodium phosphate, and the cyclic AMP content was determined as described above.

Assay of lactate dehydrogenase activity. Some 200 islets were collected in 50 µl of 50 mM-Tris/HCl buffer, pH 7.4, containing 5 mM-MgCl₂ and 0.25 mM-EDTA. A homogenate was prepared by sonication and the sonicate was dialysed against the same buffer. A sample of the homogenate was added to the reaction mixture which consisted of (final concentrations): 50 mM-Tris/HCl buffer, pH 7.4; 2 mM-sodium pyruvate; 0.2 mM-NADH; 1.5 mM-MgCl₂; 0.05 mM-EDTA; 0.03% human serum albumin. Incubation was carried out for 20 min at 37°C. The total incubation volume was 200 µl. The reaction was stopped by adding 20 µl of 2 M-HCl, which also destroyed all NADH in the course of 30 min at room temperature. The NAD⁺ formed was measured as the fluorescence induced by adding 200 µl of 12 M-NaOH and incubating the mixture for 30 min at 37°C. Before reading the

fluorescence the samples were diluted by the addition of 2.6ml of distilled water. Blanks without either pyruvate or homogenate were run in each assay.

Results

Insulin secretion

Fig. 1 shows the dose-response curve for inosine-stimulated insulin secretion. In the absence of glucose as little as 2.5mM-inosine significantly stimulates secretion in islets from fed mice ($P < 0.01$). A maximum secretory response is seen at 5mM-inosine. Further addition of inosine has no effect on the insulin secretion. Starvation decreases the inosine-induced insulin secretion totally. Table 1 shows the insulin secretion when inosine is supplemented with glucose or some modifiers of glucose-induced insulin secretion. Glucose (5mM) potentiates the effect of 5mM-inosine both in islets from fed and starved mice (although not statistically significant in the fed state). Caffeine (5mM) also potentiates the secretory response to inosine, and it restores the decreased insulin secretion seen during starvation completely. Mannoheptulose, which inhibits glucose-stimulated insulin release, presumably by interaction with the glucose-phosphorylating enzymes, has a potentiating effect on inosine-induced secretion in islets from fed mice. It is well documented that ribose does not stimulate release in mouse islets (Ashcroft *et al.*, 1972). In our system neither 10mM-ribose nor 10mM-hypoxanthine affected insulin release (results not shown).

Fig. 1. Dose-response curve for inosine-induced insulin secretion

Batches of five islets from fed and 48 h-starved mice were incubated for 2 h at 37°C in 0.3ml of the gassed Krebs bicarbonate buffer containing human serum albumin (2mg/ml) and inosine at the given concentrations. Results are given as the means $\pm$ S.E.M. of 20 batches of islets. ●, Islets from fed mice; ○, islets from 48 h-starved mice.

Inosine metabolism

As the ability of inosine to function as a primary stimulus of insulin secretion may be due not to inosine itself, but to a metabolic product of inosine formed during incubation, we have tried to determine whether inosine is in fact metabolized in the islets. It was possible to demonstrate a nucleoside phosphorylase in homogenates of islets which is capable of splitting inosine to hypoxanthine and ribose 1-phosphate. The activity of this enzyme was about 9.7 ± 0.4 nmol/h per μ g of DNA (mean $\pm$ S.E.M., $n = 5$) in islets from fed mice and 8.9 ± 0.7 nmol/h per μ g of DNA (mean $\pm$ S.E.M., $n = 5$) in islets from starved mice. The difference between the fed and starved state was not statistically significant. This activity is of the same order of magnitude as the glucose-phosphorylating capacity in mouse islets (Hedekov & Capito, 1974). If the ribose part of inosine is further metabolized through the pentose phosphate cycle, a rise in the concentration of the glycolytic intermediates and an increased production of lactate would be expected. Table 2 shows that the content of (fructose 1,6-diphosphate + triose phosphates) is raised about threefold in islets from fed mice and about fivefold in islets from starved mice. The concentration of (fructose 1,6-diphos-

Table 1. Effect of glucose, caffeine and mannoheptulose on inosine-stimulated insulin secretion

Batches of five islets from fed and 48 h-starved mice were incubated for 2 h at 37°C in 0.3ml of the gassed Krebs bicarbonate buffer containing human serum albumin (2mg/ml) and other additions as given. The released insulin was determined by radioimmunoassay as described in the Experimental section. The results are given as means $\pm$ S.E.M. for 15-20 batches of islets. A unit is 40 μ g of insulin. For assessment of statistical significance ordinary Student's *t* test has been applied.

Additions	Insulin release (μ units/ml per 2 h) per five islets	
	Fed	Starved
None	379 $\pm$ 45	357 $\pm$ 56
5mM-Glucose	358 $\pm$ 38	382 $\pm$ 41
5mM-Inosine	943 $\pm$ 98	337 $\pm$ 41
5mM-Inosine + 5mM-glucose	1508 $\pm$ 230	1018 $\pm$ 176****
10mM-Inosine	795 $\pm$ 52	360 $\pm$ 40
10mM-Inosine + 5mM-caffeine	1717 $\pm$ 194**	1509 $\pm$ 118***
10mM-Inosine + mannoheptulose (3mg/ml)	1085 $\pm$ 107**	438 $\pm$ 44*

* $P > 0.05$ versus 10mM-inosine control, not significant; ** $P < 0.05$ versus 10mM-inosine control; *** $P < 0.001$ versus 10mM-inosine control; **** $P < 0.05$ versus 5mM-inosine control.

Table 2. *Effect of starvation on the concentration of glucose 6-phosphate, (fructose 1,6-diphosphate+triose phosphates), ATP and cyclic AMP*

The metabolic parameters were measured as described in the Experimental section. For determination of ATP, glucose 6-phosphate, (fructose diphosphate + triose phosphates) and cyclic AMP, islets were incubated for 30 min at 37°C in pure Krebs bicarbonate buffer or in Krebs bicarbonate buffer containing 10 mM-inosine. Results are given as means $\pm$ S.E.M. with the numbers of determinations in parentheses. Statistical evaluation of differences has been performed as described in Table 1. * $P < 0.01$ versus control; † $P < 0.005$ versus fed inosine value; †† $P < 0.001$ versus fed inosine value; ** $P < 0.005$ versus control; *** $P < 0.001$ versus control; ††† $P < 0.005$ versus fed control value.

Metabolic parameter	Fed		48 h-starved	
	Control	10 mM-Inosine	Control	10 mM-Inosine
Glucose 6-phosphate (pmol/10 islets)	4.5 $\pm$ 0.5 (24)	7.5 $\pm$ 0.6 (23)***	2.4 $\pm$ 0.4 (31)†††	4.9 $\pm$ 0.4 (31)***††
Fructose diphosphate + triose phosphates (pmol/10 islets)	0.09 $\pm$ 0.02 (12)	0.28 $\pm$ 0.03 (15)*	0.14 $\pm$ 0.06 (9)	0.67 $\pm$ 0.12 (11)*†
ATP (pmol/islets)	7.2 $\pm$ 0.3 (23)	11.2 $\pm$ 0.5 (24)*	8.1 $\pm$ 0.5 (14)	10.7 $\pm$ 0.2 (15)*
Cyclic AMP (pmol/10 islets)	0.070 $\pm$ 0.010 (27)	0.101 $\pm$ 0.009 (29)**	0.083 $\pm$ 0.010 (20)	0.087 $\pm$ 0.008 (20)

phate+triose phosphates) that is established after inosine stimulation is significantly higher in islets from starved mice than in islets from fed mice. The content of glucose 6-phosphate is increased approximately 100% in islets from both fed and starved mice and is significantly higher in the fed state in both control and inosine incubations. During the 2 h-incubation period 10 mM-inosine caused so little glucose production that the glucose concentration in the medium never exceeded 10 μ M in the fed or the starved state. In two experiments 10 mM-inosine decreased the amount of $^{14}\text{CO}_2$ produced from [6- ^{14}C]glucose during 90 min of incubation at 37°C by 15–20% in the starved state, but not in the fed state (results not shown).

Lactate output from islets from fed mice was 163 ± 31 pmol/2 h per 10 islets (mean $\pm$ S.E.M., $n = 8$), which is about one-half of the lactate output seen after incubation with 16.7 mM-glucose (Hedekov & Capito, 1974). After starvation inosine-induced lactate production was decreased approx. 70% to a value of 52 ± 25 pmol/2 h per 10 islets ($P < 0.025$, mean $\pm$ S.E.M., $n = 6$). The maximally extractable lactate dehydrogenase activity was not changed by starvation being 7.4 ± 2.0 nmol/h per μ g of DNA (mean $\pm$ S.E.M., $n = 4$) in the fed state and 8.6 ± 1.6 nmol/h per μ g of DNA (mean $\pm$ S.E.M., $n = 4$) in the starved state. As shown in Table 2, inosine prevents the fall in ATP content normally seen when the islets are incubated without glucose (Ashcroft *et al.*, 1973b). Inosine established a significantly higher ATP content in islets from both fed and starved mice than that found in the control islets.

We have demonstrated (Capito & Hedekov, 1974) that glucose increases the concentration of cyclic AMP in islets from fed mice, but not in islets from starved mice. This phenomenon is also observed after

incubation with 10 mM-inosine. Inosine increases the content of cyclic AMP by 44% in islets from fed mice, whereas virtually no change is seen in islets from starved mice. In two experiments 10 mM-inosine did not affect adenylate cyclase activity in homogenates of islets from either fed or starved mice (results not shown).

In all experiments mentioned above, control incubations were made with 10 mM-hypoxanthine instead of 10 mM-inosine. In no instance were the measured parameters influenced by the addition of hypoxanthine.

Discussion

To our knowledge inosine has not previously been shown to be a primary stimulus of insulin secretion. Feldman & Jackson (1974) showed that 10 mM-adenosine stimulates insulin secretion, whereas uridine has no effect on secretion. During starvation inosine-stimulated insulin secretion was depressed, but when 5 mM-caffeine was added to the medium the impaired insulin response was restored. The mechanism of this restoring capacity of caffeine is discussed in more detail elsewhere (Hedekov & Capito, 1975).

Feldman & Jackson (1974) have proposed that adenosine stimulates insulin secretion by being converted into ATP. It is hardly likely that such a mechanism underlies the inosine stimulation since inosine cannot be phosphorylated to IMP (Whittam & Wiley, 1967). Tables 1 and 2 show that no correlation exists between the ATP content in the islets and the insulin secretion.

Inosine might have a direct effect on calcium translocation in islets. However, Howell & Montague (1975) were unable to find any effect of adenosine on ^{45}Ca accumulation in homogenates of rat islets

and, taking into account the structural analogy between inosine and adenosine, this possibility seems very remote.

We rather favour the idea that the stimulatory effect of inosine is due to a metabolic product of the nucleoside. This assumption is based on the alterations in islet metabolism that are found in the fed state after incubation with inosine. The necessary nucleoside phosphorylase activity is present; lactate production can be demonstrated, the content of (fructose 1,6-diphosphate+triose phosphates) and of glucose 6-phosphate is raised 300 and 65% respectively, and the ATP content is about 50% higher in islets incubated with 10mM-inosine than in control islets. Inosine (10mM) also causes glucose production in the islets which, however, is so slight that stimulatory concentrations of glucose in the medium can not be reached during a 2h incubation.

We have shown (Capito & Hedekov, 1974) that glucose increases the concentration of cyclic AMP in islets from fed mice but not in islets from starved mice, and concluded that the effect was not due to glucose itself, but to a glucose metabolite. Inosine (10mM) causes a rise in cyclic AMP content similar to that seen with 17mM-glucose, but again the effect is only observed in islets from fed mice. As the inosine-induced lactate production is depressed during starvation, and the concentrations of (fructose 1,6-diphosphate+triose phosphates) are significantly higher than in the fed state, it is tempting to speculate that the failure of inosine to raise the concentration of cyclic AMP in islets from starved mice may also be due to decreased formation of a glycolytic intermediate below the triose phosphate level, or to a decreased reoxidation of NADH.

Both glucose-induced and inosine-induced insulin release is impaired during starvation. The impairment is in both cases correlated with a decreased lactate formation (Hedekov & Capito, 1974). However, as opposed to when glucose is the stimulus, inosine produces a higher content of (fructose 1,6-diphosphate+triose phosphates) during starvation than that seen in the islets from fed mice. A decreased affinity for glucose of the hexose phosphorylating enzymes has been demonstrated in islets from starved mice (Hedekov & Capito, 1974), but as the suggested pathway for inosine metabolism circumvent the hexokinase-glucokinase step, these enzymes can not be the ones that are regulated by starvation in the inosine experiments.

The results seem to indicate that some enzyme step in the glycolytic pathway below the triose phosphate level, e.g. glyceraldehyde 3-phosphate dehydrogenase, is sensitive to starvation when inosine is the stimulus and that initiation of insulin release by inosine may depend on the production of a metabolic signal below the triose phosphate level, or a sufficient reoxidation of NADH. This suggestion

agrees with the observed correlation between glucose-induced insulin secretion and the availability of NAD⁺ in the cells (Deery & Taylor, 1973), and with the suggestion put forward by Hellman *et al.* (1974) that oxidation of glyceraldehyde 3-phosphate may be rate-limiting for insulin secretion. Decreased activity of glyceraldehyde 3-phosphate dehydrogenase may be due to either a decreased amount of enzyme or a diminished reoxidation of NADH by the α -glycerophosphate shuttle, other shuttles or by lactate dehydrogenase. From our experiments it can only be said that the maximally extractable amount of lactate dehydrogenase is not diminished during starvation.

Mannoheptulose has a slight potentiating effect on the inosine-induced insulin release. A similar effect of mannoheptulose is seen, when insulin secretion is induced by glyceraldehyde (Ashcroft *et al.*, 1973b; Hellman *et al.*, 1974), which speaks in favour of the proposed mode of action of inosine. The mechanism of this potentiation by mannoheptulose is not known, but may be related to a decreased oxidation of pyruvate (Hedekov *et al.*, 1972).

The present results do not enable us to exclude completely that inosine itself has a direct effect on a membrane receptor in the islets, but the fact that neither of the two constituents of the inosine molecule (ribose and hypoxanthine) stimulated insulin release makes it seem very unlikely that inosine itself would bind to a membrane receptor and thereby trigger insulin release. On the contrary, the many striking similarities between the effects of glucose, glyceraldehyde and inosine on insulin secretion and islet metabolism suggest that by being metabolized and furnishing glycolytic intermediates inosine acts as a functional analogue of glucose as a secretory stimulus.

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