Ascorbic acid is essential for the release of insulin from scorbutic guinea pig pancreatic islets

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ABSTRACT Pancreatic islets from young normal and scorbutic male guinea pigs were examined for their ability to release insulin when stimulated with elevated D-glucose. Islets from normal guinea pigs released insulin in a D-glucose-dependent manner showing a rapid initial secretion phase and three secondary secretion waves during a 120-min period. Islets from scorbutic guinea pigs failed to release insulin during the immediate period, and only delayed and decreased responses were observed over the 40–60 min after D-glucose elevation. Insulin release from scorbutic islets was greatly elevated if 5 mM L-ascorbic acid 2-phosphate was supplemented in the perfusion medium during the last 60 min of perfusion. When 5 mM L-ascorbic acid 2-phosphate was added to the perfusion medium concurrently with elevation of medium D-glucose, islets from scorbutic guinea pigs released insulin as rapidly as control guinea pig islets and to a somewhat greater extent. L-Ascorbic acid 2-phosphate without elevated D-glucose had no effect on insulin release by islets from normal or scorbutic guinea pigs. The pancreas from scorbutic guinea pigs contained 2.4 times more insulin than that from control guinea pigs, suggesting that the decreased insulin release from the scorbutic islets was not due to decreased insulin synthesis but due to abnormal insulin secretion.

Scorbutic guinea pigs have a depressed ability to release insulin from pancreatic islets when stimulated with glucose (1–3). This condition is characterized by lowered glucose tolerance (1, 2), degranulation of the β cells (3), and decreased deposition of glycogen in the liver (2), attributed to decreased pancreatic insulin content (2). All symptoms of abnormal insulin hypofunction are alleviated by treatment of the deficient guinea pigs with ascorbic acid. Banerjee *et al.* (4) observed a decrease in glutathione (GSH) and an increase in dehydroascorbic acid in the pancreas of scorbutic guinea pigs. Thus, ascorbic acid and perhaps GSH are implicated in the regulation of insulin biosynthesis and/or release; yet, the mechanism is unknown.

In recent studies, it was shown that protein disulfide isomerase (PDI) has intrinsic dehydroascorbate reductase activity (5). A model was proposed in which dehydroascorbic acid would cyclically act as an oxidant in the PDI reactions (6). That is, the oxidation of intracellular ascorbic acid by a hypothetical oxidase or peroxidase was postulated to occur at the surface of the endoplasmic reticulum in cells undergoing secretory protein synthesis despite the presence of a high cytoplasmic GSH/oxidized glutathione (GSSG) ratio (7). The resulting dehydroascorbic acid, moving across the endoplasmic reticulum membrane, would oxidize the active center cysteines of PDI, as shown by Venetianer and Straub (8, 9) and Givol *et al.* (10), which would in turn oxidize the nascent protein sulfhydryl groups for the native disulfide conformation. Ascorbic acid, reformed from the reaction with PDI, would diffuse back into the cytoplasmic space, and the reduced PDI would be reoxi-

dized by freshly derived dehydroascorbic acid. In the absence of ascorbate, therefore, cells may synthesize the protein precursors lacking disulfide formation. To test this hypothesis, we compared the ability of isolated pancreatic islets from normal and scorbutic guinea pigs to release insulin as a response to perfused glucose, *in vitro*, in the presence and absence of ascorbic acid as the 2-phosphate. The 2-phosphate group stabilizes ascorbic acid (11). The derivative is susceptible to plasma membrane alkaline phosphatase activity (12), thus releasing metabolically active ascorbic acid for cellular uptake either as ascorbic acid directly or as dehydroascorbic acid after extracellular oxidation (13). In the studies reported herein, we demonstrate the abnormal release of insulin by pancreatic islets from scorbutic guinea pigs and show, by direct measurement, *in vitro*, that ascorbic acid, administered as the precursor, L-ascorbic acid 2-phosphate, stimulates the release of insulin from scorbutic guinea pig islets in the presence of elevated glucose.

EXPERIMENTAL PROCEDURES

Materials. Guinea pigs were purchased from the Michigan Department of Public Health and Charles River Breeding Laboratories. Ascorbic acid-free diet for guinea pigs (ascorbic acid test–guinea pig) and bovine serum albumin (BSA; RIA grade) were purchased from United States Biochemical. Ascorbic acid, collagenase type V, chicken egg albumin, guanidine hydrochloride, anti-rabbit IgG (alkaline phosphatase conjugated), *p*-nitrophenyl phosphate, 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, bisbenzamide, calf deoxyribonucleic acid, and DEAE-Sephadex were purchased from Sigma. Sephadex G-50 was purchased from Pharmacia LKB. Bio-Gel P-2, Bio-Gel P-30 acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate, SDS, and Coomassie brilliant blue R-250 were purchased from Bio-Rad. Nylon mesh (10 μ m, pore size) was purchased from Whatman. Standard guinea pig insulin and anti-guinea pig serum were kindly provided by Cecil C. Yip (University of Toronto). Guinea pig pancreas was purchased from Rockland (Gilbertsville, PA). Difluorodinitrobenzene was a product of Pierce.

Scorbutic Guinea Pigs. Male weanling guinea pigs were 150–180 g at the beginning of the feeding periods. Control and scorbutic animals were fed the same commercial ascorbic acid-free diet, *ad libitum*, throughout the experiments. The control animals received ascorbic acid in their drinking water prepared daily in the form of a 0.1% solution neutralized with sodium hydroxide to pH 7.0. Animals were weighed weekly. Symptoms of weight loss and difficult movements were seen typically between 21 and 25 days after initiation of the dietary protocol in the animals not supplemented with ascorbate. All animals were fasted overnight prior to the preparation of islets.

Isolation of Islets. Islets were immediately isolated from guinea pig pancreas by collagenase treatment by the proce-

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Abbreviations: GSH/GSSG, reduced/oxidized glutathione; BSA, bovine serum albumin.

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dures of Wollheim *et al.* (14) and Gardner and Jackson (15). Individual islets were hand-picked from the digest mixture in Krebs–Ringer bicarbonate (KRB) buffer with 0.1% BSA by using a Pipetman with the aid of a Leica dissection microscope.

Islet Perfusions. A typical preparation of 40–60 islets from either control or scorbutic guinea pigs was placed in a chamber made from a plastic 2-ml syringe and sandwiched between 300 μ l of a Bio-Gel P-2 slurry in the perfusion buffer (KRB). The buffers contained 118 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4 , 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 5 mM NaHCO_3 , and 10 mM Hepes (pH 7.4). The perfusion buffer consisted of KRB with 0.1% BSA. The solution was equilibrated with a mixture of 95% O_2 /5% CO_2 for 15 min and adjusted to pH 7.4 before perfusion.

Tubing was connected to the sealed chamber immersed in a waterbath at 37°C. In the first experimental series, buffer containing 1.7 mM D-glucose was perfused at a rate of 0.5 ml/min. Elevation of glucose concentration to 20 mM and addition of 5 mM ascorbic acid 2-phosphate were done at the indicated time of perfusion (see Figs. 2–4). Fractions of the perfusate were collected at 5-min intervals and were immediately frozen and stored at -70°C until analyzed for insulin content by an ELISA procedure.

DNA Analysis. To avoid error due to variation in islet size or protein contamination from the BSA-containing medium, we expressed the released insulin on the basis of islet DNA. The contents of the chamber (gel and islets) were suspended in 2 ml of 50 mM sodium phosphate, pH 7.4/2 M NaCl in a plastic tube and homogenized with a Tekmar (Cincinnati) Tissumiser at 70 V for 2 min at 4°C. The gel slurry was removed by centrifugation of the sample through a 0.22- μ m (pore size) filter-containing centrifuge tube (Costar Spin-X). The DNA content was determined fluorimetrically by the method of Labarca and Paigen (16).

Guinea Pig Insulin Isolation. Insulin was isolated from frozen pancreas obtained from Rockland by the methods of Zimmerman and Yip (17) and Treacy *et al.* (18). The purification was monitored by dot blot analysis of fractions using rabbit anti-guinea pig serum and a secondary anti-rabbit antibody conjugated with alkaline phosphatase.

Insulin Conjugation and ELISA Analysis. Guinea pig insulin was conjugated with chicken egg albumin as described by Tager (19) to assure binding to 96-well plates. The conjugated guinea pig insulin was purified and stored at -70°C until used for a competitive ELISA analysis after the general procedure of Bank (20). The data were calculated from the standard curve plots and expressed as ng of insulin released per 5-min fraction per μ g of islet DNA.

Pancreatic Ascorbic Acid, GSH + GSSG, and Insulin Analysis. The ascorbic acid content of control and scorbutic guinea pig pancreas was assayed by homogenization of weighed samples of tissue in 2 ml of 10% (vol/vol) metaphosphoric acid/1 mM thiourea/1 mM EDTA. Aliquots of the whole homogenate were taken for protein analysis by the bicinchoninic acid protein assay protocol according to the manufacturer's direction (Pierce) with BSA as standard. The remainder of the homogenate was centrifuged and diluted 1:50 with homogenizing fluid for samples from control animals. The samples from ascorbic acid-deficient guinea pigs were directly analyzed. Ascorbic acid content for both samples was analyzed as described (21). For the analysis of total pancreatic GSH + GSSH, weighed samples of pancreas were homogenized in 5% (wt/vol) sulfosalicylic acid. Aliquots were taken for protein analysis as described above. Other aliquots were centrifuged and total GSH + GSSG concentration was determined by the method of Roberts and Francetic (22). For the analysis of pancreatic insulin, 200 mg of guinea pig pancreas was homogenized in 2 ml of acidified ethanol by using a Tekmar Tissumiser (17). The homogenate was centrifuged at $4000 \times g$ for 30 min and the resulting supernatant fraction was further centrifuged at $10,000 \times g$ for 30 min. The proteins in

the supernatant equivalent to those from 1 mg of fresh tissue were separated by SDS/PAGE under the reducing conditions described by Ito *et al.* (23). The separated proteins were then electroblotted onto nitrocellulose membranes (0.2 μ m, pore size) for 2 hr at 70 V. The insulin bands (A and B chains) were visualized by rabbit anti-guinea pig insulin antibody, anti-rabbit IgG antibody (conjugated with alkaline phosphatase), 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium and quantitated by densitometry (densitometer package SW 2000; Ultraviolet Products, San Gabriel, CA).

Ascorbic Acid 2-Phosphate Dephosphorylation by Pancreatic Islets. To determine the extent to which typical islet preparations hydrolyze ascorbic acid 2-phosphate, islets from normal and scorbutic guinea pigs were preincubated in 1.0 ml of incubation buffer containing 1.7 mM glucose and equilibrated with 95% O_2 /5% CO_2 at 37°C for 15 min. The incubation continued for 30 min in the same medium containing 20 mM glucose and 5 mM ascorbic acid 2-phosphate. The incubation mixture was rapidly centrifuged, and 1.0 ml of 10% metaphosphoric acid/1 mM thiourea/1 mM EDTA was added to the pellet with homogenization. Aliquots of the homogenate were analyzed for DNA by the method of Labarca and Paigen (16). To the supernatant, an equal volume of 20% metaphosphoric acid/2 mM thiourea/2 mM EDTA was added. This solution was centrifuged to remove precipitated serum albumin and any solubilized cellular protein. The ascorbic acid in each solution was analyzed by HPLC and electrochemical detection as described (21). Control medium without islets was analyzed simultaneously.

Electron Microscopy. Isolated islets from control and scorbutic guinea pig pancreas were quickly fixed in 3% (vol/vol) glutaraldehyde in 0.05 M cacodylate buffer (pH 6.8). The samples were post-fixed in buffered 1% osmium tetroxide and stained in half-saturated uranyl acetate. After dehydration, the samples were infiltrated with epoxy resin. Ultrathin sections (85 nm) were cut, mounted on 300-mesh copper grids, and stained with uranyl acetate and lead citrate. The grids were carbon-coated to stabilize the sections, which were viewed at 100 kV with a JEOL 100CX II Temscan electron microscope.

Statistics. Statistical analysis of the mean $\pm$ SD was conducted with the aid of computer software, INSTAT, from GraphPad (San Diego).

RESULTS

Pancreatic Insulin, Ascorbic Acid, and Total GSH + GSSH.

The ascorbic acid content of pancreas from scorbutic guinea pigs was $\approx 1\%$ that of the control animals (Table 1), verifying the severe ascorbic acid deficiency observed by other criteria such as measurement of body weight and difficulty of mobility. In the pancreas from scorbutic guinea pigs, the total GSH + GSSG was $\approx 70\%$ that of the control animals. The insulin content of the pancreas from scorbutic guinea pigs showed some variation, two of four being similar to control values

Table 1. Comparison of total GSH + GSSH, ascorbic acid, and insulin content of pancreas from normal and scorbutic guinea pigs

Animals	GSH + GSSG, nmol/mg of protein	Ascorbic acid, μ mol/g of protein	Insulin, units
Control	11.02 ± 3.34	7.34 ± 1.40	66 ± 32
Scorbutic	7.70 ± 3.04	$0.08 \pm 0.02^*$	158 ± 102

Eight male guinea pigs 200–250 g were fed a scorbutogenic diet for 25–28 days. The control group received daily supplements of 0.1% neutralized ascorbic acid in the drinking water. The pancreas was rapidly removed after euthanasia and samples of tissue were removed and assayed for ascorbic acid, total GSH + GSSG, and insulin. Insulin is expressed as relative intensity units measured by an Ultraviolet Products densitometer. ($n = 4$.)

*Statistically different from control animals; $P < 0.0001$ ($n = 4$).

FIG. 1. Western blot analysis of insulin from equal aliquots of control guinea pig pancreas (lanes 1–4) and scorbutic guinea pig pancreas (lanes 5–8).

whereas two others were significantly higher. The mean insulin content was 2.4 times higher than that of the control guinea pigs (Table 1 and Fig. 1).

Insulin Release from Guinea Pig Islet Cells in Response to Glucose Perfusion: Effect of Ascorbic Acid. The insulin release by pancreatic islets from normal guinea pigs in response to glucose elevation was immediate with an initial phase followed by secondary waves of insulin secretion over the succeeding 120 min (Fig. 2). In contrast, pancreatic islets from scorbutic guinea pigs failed to respond immediately to glucose elevation. A delayed or secondary response (70–80 min) was significantly lower than that of the equivalent phase of release for control islet preparations. In the second series (Fig. 3), a pattern similar to the first series was seen for normal guinea pig islets—i.e., 5 mM ascorbic acid 2-phosphate had no effect on insulin release. However, scorbutic guinea pig islets responded to 20 mM D-glucose with increased insulin secretion only after 5 mM ascorbic acid 2-phosphate was added at perfusion time, 90 min. The results in Fig. 4 show that pancreatic islets from scorbutic guinea pigs responded as rapidly as the controls with elevated insulin release when ascorbic acid 2-phosphate was supplemented simultaneously with the elevation of glucose levels to 20 mM at perfusion time, 30 min. Islets from scorbutic guinea pigs released increased amounts of insulin compared with controls especially during the secondary release waves, suggesting they had accumulated elevated insulin deposits in preparation for a future glucose-mediated signal event, a signal that somehow requires ascorbic acid. When the 1.7 mM D-glucose level of the perfusion medium was maintained over the entire incubation period, and 5 mM ascorbic acid 2-phosphate was supplemented after the first 30 min, no release of insulin from islets from control or scorbutic animals occurred over the next 120 min (data not shown).

Ascorbic Acid 2-Phosphate Dephosphorylation by Pancreatic Islets. The islet-cell membrane phosphatase activity in the

FIG. 2. Comparison of the effects of elevated D-glucose (20 mM) on insulin release from pancreatic islets of normal (●) and scorbutic (○) guinea pigs. Perifusions were initiated with KRB medium supplemented with 0.1% BSA and 1.7 mM D-glucose at a rate of 0.5 ml/min at 37°C. D-Glucose was increased to 20 mM after 30 min (arrow), followed by collection of fractions for 120 min. Values are the mean $\pm$ SD ($n = 3$).

FIG. 3. Comparison of the effects of elevated D-glucose and delayed ascorbic acid 2-phosphate on insulin release from pancreatic islets of normal (●) and scorbutic (○) guinea pigs. Perifusions were initiated with KRB medium supplemented with 0.1% BSA and 1.7 mM D-glucose at a rate of 0.5 ml/min and 37°C. D-Glucose was increased to 20 mM after 30 min (open arrow), and after 90 min, 5 mM ascorbic acid 2-phosphate was added (cross-hatched arrow). Values are the mean $\pm$ SD for normal ($n = 5$) and scorbutic ($n = 9$) animals.

incubation medium was assessed by the analysis of ascorbic acid released during a 30-min period as described above. Ascorbic acid levels in the islets from control and scorbutic guinea pigs were 0.33 and 0.04 nmol/µg of DNA, respectively. The ascorbic acid levels of the medium resulting from islet phosphatase activity were 0.78 and 0.26 nmol per ml per µg of DNA, respectively. The concentration of ascorbic acid was estimated to be 3.0–5.0 µM and dependent on the number and size of islets. Thus, the islets from scorbutic guinea pigs (Figs. 3 and 4) might require only micromolar levels of ascorbic acid for expression of the glucose (20 mM)-stimulated insulin release.

Electron Microscopy. Electron microscopic evidence (Fig. 5) supports the concept that insulin is present in equal or higher amounts in the islets from scorbutic animals compared with islets from normal guinea pigs, in agreement with the data obtained by the Western blot analysis (Fig. 1).

DISCUSSION

The present study confirms the original observations of Sigal and King (1) and Banerjee and coworkers (2–4) that the release of insulin from pancreatic islets of scorbutic guinea pigs

FIG. 4. Comparison of the effects of elevated D-glucose (20 mM) and L-ascorbic acid 2-phosphate (5 mM) on insulin release from pancreatic islets of normal (●) and scorbutic (○) guinea pigs. Perifusions were initiated with KRB medium supplemented with 0.1% BSA and 1.7 mM D-glucose at a rate of 0.5 ml/min and 37°C. D-Glucose was increased to 20 mM concurrently with 5 mM ascorbic acid 2-phosphate after 30 min (arrow). Values are the mean $\pm$ SD for normal ($n = 3$) and scorbutic ($n = 5$) animals.

FIG. 5. Electron micrographs of β cells from normal (*A*) and scorbutic (*B*) guinea pigs. (Bar = 720 nm.) Insulin-containing secretory granules in normal cells (*A*) are typically close to the plasma membrane (arrows), whereas those from a vitamin C-deficient cell (*B*) are mostly repressed from the plasma membrane (arrows).

is impaired. However, we conclude, in contrast to Banerjee (2), that the biosynthesis of insulin is not ascorbic acid-dependent but, rather, that ascorbic acid is necessary to mediate the D-glucose-stimulated release of insulin. The discrepancy between our data and Banerjee's data (2, 3) relative to insulin content of scorbutic guinea pig pancreas may be explained by the method used by Banerjee (3), namely, a hypoglycemia bioassay of guinea pig pancreas extracts in rabbits. Hypoglycemia bioassay of guinea pig insulin in rabbits may have been unsuitable considering the currently known wide differences in chemical structure and immunological properties of guinea pig insulin compared with many other species (17, 24).

Megadoses of vitamin C delayed insulin response to a glucose challenge in normoglycemic human adults, suggesting possible conflicts between elevated plasma ascorbic acid and

glucose transport across β -cell membranes (25). In a recent study, the inhibitory effect of ascorbic acid on insulin release from single rat pancreatic islets was reported (26) in which 50% inhibition of insulin secretion from normal rat islets occurred at 200 μ M ascorbic acid. In addition, the ascorbic acid content of the rat islets used in the previous study (26) was 4.4 mM, whereas our pancreatic ascorbic acid values, expressed as nmol/g of protein, were 0.08 ± 0.02 . This amount is $\approx 1\%$ of that in control guinea pig pancreas. We chose the long-lasting inert provitamin C, ascorbic acid 2-phosphate, which was hydrolyzed to low levels of ascorbate (3–5 μ M) presumably by islet phosphatase activity. This difference may explain why we observed the effect of ascorbic acid deficiency on the D-glucose-dependent release of insulin from pancreatic islets and demonstrated a requirement for ascorbic acid in

glucose-mediated insulin release. The previous study (26) of inhibition of insulin secretion by levels of ascorbic acid >200 μ M, however, may provide an explanation for why plasma ascorbate concentrations are normally tightly regulated.

The use of ascorbic acid 2-phosphate was crucial in the present study since perfusion of ascorbic acid at low concentrations in the presence of trace transition metals and a pH of 7.4 would likely have resulted in rapid oxidation of ascorbic acid to dehydroascorbic acid (27) and further degradation products (28). In addition, Pence and Mennear (29) have reported the inhibitory effect of 2 mg % ($\approx 114 \mu$ M) dehydroascorbic acid on insulin secretion from mouse pancreatic islets. Although dehydroascorbic acid levels were not analyzed in the present study, we believe that since the potential source, ascorbic acid, was present at such low levels, the dehydroascorbic acid was likewise very minimal.

The decreased phosphatase activity of islets from scorbutic animals, compared with normal animals using ascorbic acid 2-phosphate as substrate, is reminiscent of an old observation that the alkaline phosphatase activity of the plasma of infants and young children suffering from scurvy was low (30). This was also seen by several workers in guinea pigs with attempts made to rule out inanition by use of the paired-feeding technique (31). The protocol used for all the present perfusion experiments included uniformly oxygen-equilibrated medium ruling out potential adverse effects of variable oxygen environments known to affect insulin secretion from pancreatic islets (32). Our present results are consistent with a possible role for modification of the redox state of the NADPH/NADP and GSH/GSSG systems modulated by entry of D-glucose into the β cells (33), since a decreased but not statistically significant total GSH + GSSH level was observed in pancreas from scorbutic animals compared with those from normal animals.

Our original proposal for a role of an ascorbic acid redox cycle in protein disulfide formation using insulin biosynthesis as a model is not supported by the present observations. We have examined the insulin in the pancreas of scorbutic guinea pigs by SDS/PAGE under reducing and nonreducing conditions. No reduced insulin was detected in scorbutic guinea pigs under nonreducing SDS/PAGE, and no proinsulin, which might have accumulated if the proper disulfide bond formation was defective, was detected. Instead, the present work identified a function for ascorbic acid in enhancing the competency of glucose in the activation cascade of insulin release (34).

The broad elements of glucose-induced insulin secretion have been reviewed extensively (35–37). Glucose-induced insulin release results from vesicular exocytosis, a process that is triggered by the entry of Ca^{2+} across the plasma membrane (38, 39). Ca^{2+} is known to enter through voltage-sensitive calcium channels. Accordingly, glucose must generate a signal that involves the depolarization of the β -cell membrane. Current studies reveal a synergistic interaction in β cells between the glucose-regulated ATP-dependent signaling system and the hormonally regulated cAMP-dependent signaling system. This interaction gives β cells the ability to match the ambient concentration of glucose to an appropriate insulin secretory response, a process referred to by Holz and Habener (39) as the induction of glucose competence. Recently, ascorbic acid has been reported to modulate calcium channels in pancreatic β cells by inactivating ($\text{IC}_{50} = 1 \text{ mM}$) the slow deactivating calcium channels (40). The observed effect of ascorbic acid requires metal ions, suggesting mediation by the oxidation product, dehydroascorbic acid, previously known to inhibit insulin release (28). Our results offer an explanation for the old observations of Sigal and King (1) and Banerjee and coworkers (2–4) that demonstrated an abnormal response of scorbutic guinea pigs to D-glucose-stimulated insulin release.

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