Original Article

Zinc, Not Insulin, Regulates the Rat α-Cell Response to Hypoglycemia In Vivo

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The intraislet insulin hypothesis proposes that the decrement in β-cell insulin secretion during hypoglycemia provides an activation signal for α-cells to release glucagon. A more recent hypothesis proposes that zinc atoms suppress glucagon secretion via their ability to open α-cell ATP-sensitive K^+ channels. Since insulin binds zinc, and zinc is cosecreted with insulin, we tested whether decreased zinc delivery to the α-cell activates glucagon secretion. In streptozotocin-induced diabetic Wistar rats, we observed that switching off intrapancreatic artery insulin infusions in vivo during hypoglycemia greatly improved glucagon secretion (area under the curve [AUC]: control group 240 ± 261 and experimental group 4,346 ± 1,259 pg · ml^{-1} · 90 min^{-1}; $n = 5$, $P < 0.02$). Switching off pancreatic artery infusions of zinc chloride during hypoglycemia also improved the glucagon response (AUC: control group 817 ± 107 and experimental group 3,445 ± 573 pg · ml^{-1} · 90 min^{-1}; $n = 6$, $P < 0.01$). However, switching off zinc-free insulin infusions had no effect. Studies of glucose uptake in muscle and liver cell lines verified that the zinc-free insulin was biologically active. We conclude that zinc atoms, not the insulin molecule itself, provide the switch-off signal from the β-cell to the α-cell to initiate glucagon secretion during hypoglycemia. *Diabetes* 56:1107–1112, 2007

Glucagon secretion into the hepatic portal circulation is critical for counterregulation of hypoglycemia by virtue of its stimulatory effect on hepatic glycogenolysis, which releases glucose into the systemic circulation to return blood glucose to normal levels. The decrement in insulin release from β-cells has been proposed to signal the α-cell to release glucagon (1–3). Recently, we demonstrated this phenomenon directly in vivo in streptozotocin-induced diabetic rats and in vitro using isolated islets from these animals. In the in vivo studies, an insulin infusion into the pancreatic artery was switched off when the animals were made hypoglycemic by a jugular-vein infusion of insulin (4). Glucagon secretion, absent in control diabetic animals, was fully restored by this maneuver. Glucagon release was not initiated by this maneuver if saline rather than insulin was used, or if insulin was infused but not switched off. The glucagon response was not observed during normoglycemic or hyperglycemic conditions; the switch-off signal was effective only in the setting of hypoglycemia. We corroborated these findings with in vitro islet perifusion studies (5).

Recent work from several laboratories has demonstrated that zinc atoms are capable of suppressing α-cell function via activation of rat α-cell ATP-sensitive K^+ (K_{ATP}) channels (6–9). Since zinc atoms are bound by and cosecreted with insulin, we hypothesized that zinc might provide the switch-off signal during hypoglycemia. To evaluate this hypothesis, we performed in vivo studies using intrapancreatic artery infusions of zinc-containing insulin, zinc chloride alone, or zinc-free insulin in streptozotocin-induced diabetic Wistar rats. Insulin and zinc chloride provided the switch-off signal to the α-cell during hypoglycemia, but zinc-free insulin did not. These results lead us to conclude that decreased delivery of zinc, not insulin, provides the signal from the β-cell to the α-cell to secrete glucagon during hypoglycemia in the rat.

RESEARCH DESIGN AND METHODS

Male Wistar rats (200–250 g) were purchased from Charles River Laboratories. Animals were placed in rooms with a 12-h light-dark cycle and constant temperature and given free access to food and water. All experiments were approved by the PNRI Institutional Animal Care and Use Committee. Diabetes was induced by intraperitoneal injection of 120 mg/kg streptozotocin ([STZ]; Sigma, St. Louis, MO) made up in freshly prepared citrate buffer. After STZ injection, blood glucose was measured daily. Rats were considered diabetic when blood glucose exceeded 350 mg/dl on two sequential measurements. Animals that did not develop diabetes by 1 day were given a second dose of STZ. Insulin pellets (Linshin Canada, Ontario, Canada) delivering ~1 unit/24 h were inserted under the skin. Blood glucose levels of insulin-treated diabetic rats were 100–250 mg/dl. Animals that were diabetic for ≥2 weeks were used for the experiments, at which time they weighed between 300 and 350 g.

Surgical procedure. On the day of the intrapancreatic artery infusions, nonfasted animals were anesthetized by inhalation of 2% isoflurane. The right jugular vein was cannulated with PE50 polyethylene tubing (Becton Dickinson, Spark, MD) filled with heparin (500 units/ml), which was advanced to the superior vena cava. This cannula was used for intravenous insulin infusion and collection of blood samples. The upper abdomen was opened by a 3-cm vertical incision in the midline starting from the xiphoid process. The pancreas typically receives its blood supply from branches of the splenic and superior and inferior pancreaticoduodenal arteries. The hepatic artery was isolated, ligated superiorly, and punctured using a 25-gauge needle. For infusion of insulin, a microcannula (0.008 mm I.D.; Biotime, Berkeley, CA) was inserted into the hepatic artery and advanced retrograde to the origin of the pancreatic artery, which was ligated over the cannula. The positioning of the tip of the cannula is at the take-off of the superior pancreaticoduodenal artery. This position does not interfere with normal blood flow coming from the celiac artery into the pancreatic arterial blood supply, which serves to carry

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AUC, area under the curve; ATCC, American *Type Culture* Collection; FBS, fetal bovine serum; K_{ATP}, ATP-sensitive K^+; M199, Medium 199; STZ, streptozotocin.

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the solutions delivered by infusion through the micocannula. After surgery, the opened abdomen was superfused with warm saline and covered with foil to prevent drying.

Study protocols. After STZ injection and establishment of hyperglycemia for ≥2 weeks, overnight-fasted, anesthetized, diabetic animals were first studied to assess whether they had intact glucagon responses to hypoglycemia. Insulin (0.5 units/ml at 50 μl/min) was infused into the jugular vein to induce hypoglycemia, and samples were collected before and at 0, 30, 60, and 90 min thereafter for blood glucose, C-peptide, and glucagon measurements. Glucose levels were measured within 2 min of sample collection. At the end of the experiment, the jugular catheter was removed, the incision was closed, and the animals were allowed 2 weeks for recovery. Once the defective glucagon response in the diabetic animals had been established, the animals were randomly divided into groups to receive intrapancreatic artery infusions of regular insulin (Humulin), zinc chloride, or zinc-free insulin.

Approximately 2 weeks later, the surgical procedure to isolate the pancreaticoduodenal artery described above was carried out, and the anesthetized animals were rested for 30 min. Insulin (0.5 units/ml at 50 μ/min), or zinc-free insulin (0.5 units/ml at 50 μ/min) in the animals receiving zinc-free insulin pancreaticoduodenal artery infusions, was infused into the jugular vein to decrease blood glucose to ~100 mg/dl. After a basal blood sample was collected, zinc chloride (4.5 or 30 mmol/l), insulin (1.5 units/ml), or zinc-free insulin (1.5 units/ml) was infused at a rate of 50 μ/min into the superior pancreaticoduodenal artery. The zinc concentrations were chosen because 30 μmol/l was used in the studies reported by Franklin et al. (9) and 4.5 μmol/l is two times greater than the half-maximal inhibitory concentrating [IC_{50}] for activation of α-cell K_{ATP} channels (9) and equal to the amount of zinc found in 1.5 units/ml of Humulin R (10). Ten minutes later, another blood sample was collected. When blood glucose fell to <60 mg/dl, the pancreatic artery infusions were switched off, and blood was sampled at 0, 15, 30, 45, 60, and 90 min.

Assays. Plasma glucose was measured immediately using a Roche Accu-check glucometer. Blood samples were collected into heparin-coated ice-chilled tubes containing 1,000 IU/ml trasylol to prevent degradation of glucagon. Plasma levels of C-peptide and glucagon were measured using rat C-peptide and glucagon radioimmunoassay kits (Linco Research, St. Charles, MO).

Preparation of zinc-free insulin. Zinc was removed from a commercial insulin preparation (Novalog; NovoNordsk) by passing over a column of Chelex 100 (BioRad Laboratories). The zinc concentration was 19.6 μg/ml based on the manufacturer's data. We used ~12 mg Chelex 100/ml insulin solution. This is approximately a 20-fold excess of Chelex 100 resin based on the manufacturer's estimate of 0.4 meq/ml. The resin was washed with 20 volumes of PBS at 4°C before loading the insulin solution. Biological activity was assayed by intraperitoneal injection into fed mice and produced hypoglycemia greater than or equal to that produced by zinc-containing insulin.

Glucose uptake into L6 cells. Rat L6 myoblasts (American *Type Culture* Collection [ATCC] catalog #CRL-1458) were maintained at subconfluence in Dulbecco's modified Eagle's medium (ATCC catalog #30-2002) supplemented with 10% fetal bovine serum (FBS) and 1× penicillin-streptomycin (Sigma P4333) at 37°C and 5% CO_2. Cells were plated in six-well plates at a density of 1×10^6 cells/well. Once the cells reached 90% confluence, the media was switched to Dulbecco's modified Eagle's medium with 2% FBS and 1× penicillin-streptomycin. They were allowed to differentiate into myotubes for 7 days with media changes every 2nd day. After the 7th day, the differentiated cells were washed with Medium 199 (M199; Sigma) and then incubated for 3 h in M199 media containing 0.1% BSA. The myotubes were then incubated for 30 min in glucose-free Krebs-Ringer Buffer (KRB) with 0.1% BSA plus 100 nmol/l insulin (Humulin R; Lilly), 100 nmol/l zinc-free insulin, or 102 nmol/l zinc chloride, based on 0.04 mg zinc chloride/100 units insulin (10). The buffer was then switched to KRB plus 0.1% BSA containing insulin, zinc-free insulin, or zinc chloride plus 0.5 μCi/ml of 2-deoxy-^{14}C-D-glucose (300 mCi/mmol; American Radiolabeled Chemicals) for 5, 10, and 20 min. The cells were washed 3 times with ice-cold PBS and harvested in 400 μl lysis buffer (0.1% SDS, 0.2 mol/l NaOH). Glucose uptake was determined by liquid scintillation counting. Nonstimulated glucose uptake was also measured in cells cultured with KRB plus 0.1% BSA containing 0.5 μCi/ml 2-deoxy-^{14}C-D-glucose for each time point and subtracted from the stimulated uptake.

Glucose uptake into HepG2 cells. Human HepG2 liver cells (ATCC catalog #HB-8065) were maintained in Eagle's Modified Essential Medium (ATCC catalog #30-2003) supplemented with 10% FBS and 1× penicillin-streptomycin at 37°C and 5% CO_2. Cells were plated in six-well plates at a density of 0.6×10^6 cells/well. After 48 h, the cells were washed with M199 and then incubated for 3 h in M199 media containing 0.1% BSA. Following a 15-min glucose washout period in glucose-free KRB + 0.1% BSA, the cells were incubated in KRB with 0.1% BSA plus 1 μmol/l insulin (Humulin R), 1 μmol/l zinc-free insulin, or 1.02 μmol/l zinc chloride. The buffer was then switched to KRB plus 0.1% BSA containing insulin, zinc-free insulin, or zinc chloride plus 0.5 μCi/ml 2-deoxy-^{14}C-D-glucose plus 500 μmol/l cold deoxyglucose for 5, 10, and 20 min. The cells were washed 3 times with ice-cold PBS and harvested in 400 μl of lysis buffer. Glucose uptake was then determined by liquid scintillation counting. Nonstimulated glucose uptake was also measured in cells cultured with KRB plus 0.1% BSA containing 0.5 μCi/ml 2-deoxy-^{14}C-D-glucose plus 500 μmol/l cold 2-deoxyglucose for each time point and subtracted from the stimulated uptake.

Statistics. Data are presented as means ± SE. Results were analyzed using Student's t test, Wilcoxon's matched pair signed-rank test, or ANOVA, as appropriate. A P value <0.05 was considered statistically significant.

RESULTS

Before abdominal surgery, the group of animals rendered diabetic by injection of STZ had basal glucagon levels of 39 ± 5 pg/ml and small but significant glucagon responses to insulin-induced hypoglycemia (AUC during hypoglycemia 549 ± 145 pg · ml^{-1} · 90 min^{-1}; $n = 20$, $P < 0.001$).

On the day of the experiment, 2 weeks later, fasted animals underwent anesthesia, laparotomy, and hepatic artery cannulation for infusion of insulin, zinc-free insulin, or zinc chloride. Hypoglycemia was induced by infusion of insulin or zinc-free insulin (for those animals receiving intrahepatic artery infusions of zinc-free insulin) into the jugular vein. Insulin and zinc-free insulin induced hypoglycemia, and switching off the pancreatic arterial infusions of insulin, zinc, or zinc-free insulin had no effect on C-peptide levels, which were barely measurable (Fig. 1). Switching off the pancreatic arterial infusion of regular insulin in hypoglycemic animals produced a marked improvement in glucagon secretion compared with the control experiment performed 2 weeks earlier (AUC: control group 240 ± 261 pg · ml^{-1} · 90 min^{-1}; after switch off, 4,346 ± 1,259; $n = 5$, $P < 0.02$ [Fig. 2]). Switching off the arterial infusion of zinc chloride (30 or 4.5 μmol/l) also produced a marked improvement in the glucagon response to hypoglycemia (AUC: 30 μmol/l control group 178 ± 841 pg · ml^{-1} · 90 min^{-1}; after switch off, 5,020 ± 2,466; $n = 3$, $P < 0.05$ [Fig. 3]; 4.5 μmol/l control group 817 ± 107 pg · ml^{-1} · 90 min^{-1}; after switch off, 3,445 ± 573; $n = 6$, $P < 0.01$ [Fig. 4]). However, the animals that received pancreatic artery infusions of zinc-free insulin did not have improved glucagon responses to hypoglycemia (AUC: control group 632 ± 164 pg · ml^{-1} · 90 min^{-1}; after switch off, 938 ± 296; $n = 6$, P = NS [Fig. 5]). The incremental glucagon responses are summarized in Fig. 6.

The zinc-free insulin preparation we used caused hypoglycemia as effectively as zinc-containing insulin when infused into the jugular vein (Fig. 1). To further assess the biological activity of zinc-free insulin, we compared the uptake of [^{14}C]-2-deoxyglucose into L6 myocyte and HepG2 hepatocyte cell lines stimulated by regular zinc-containing insulin, zinc chloride, or zinc-free insulin. We saw no significant difference in glucose uptake between zinc-free insulin and zinc-containing insulin in L6 myocytes; the effect in HepG2 hepatocytes was even greater with zinc-free insulin (Fig. 7). Zinc chloride had a small stimulatory effect on 2-deoxyglucose uptake in L6 myocytes and a larger effect in HepG2 cells.

DISCUSSION

The intraislet insulin hypothesis proposes that insulin released from the β-cell tonically inhibits glucagon secretion and that the marked attenuation of insulin release during hypoglycemia signals the α-cell to secrete glucagon

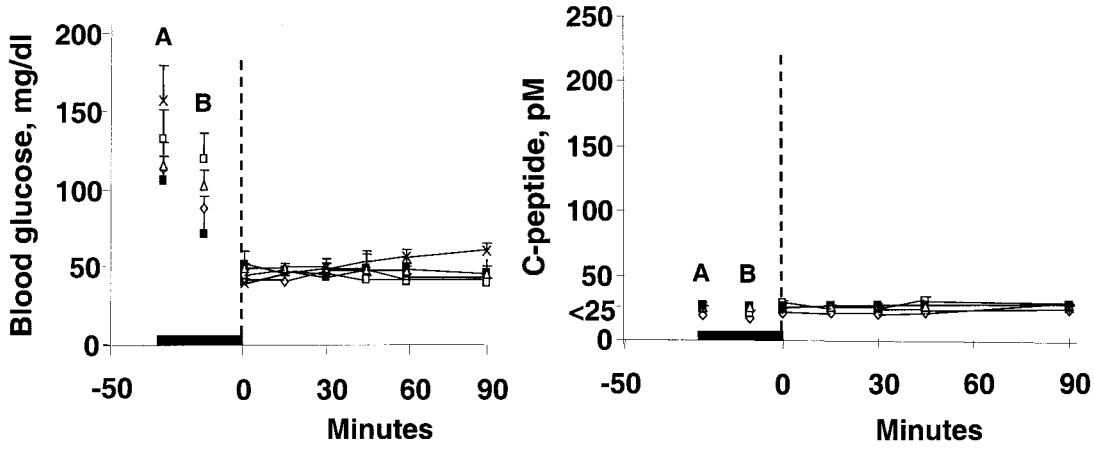

FIG. 1. Levels of glucose and C-peptide before, during, and after switching off intrapancreatic artery infusions of insulin, zinc chloride, or zinc-free insulin during hypoglycemia in STZ-induced diabetic rats. Results from the first blood sample collected during the experiments (point A) correspond with the glucose and C-peptide levels just before the jugular-vein infusions were begun to induce hypoglycemia. Intrapancreatic artery infusions (represented by the black bar along the horizontal axis) of insulin, zinc chloride, or zinc-free insulin were begun when blood glucose reached 100–150 mg/dl. Point B corresponds with the levels of glucose and C-peptide 10 min after the pancreatic artery infusions started. The vertical dashed line at 0 time indicates the levels of glucose and C-peptide at the time that the infusions were switched off. The time elapsed between point A and the switching off of the pancreatic artery infusions varied depending on how much time it took (generally 30–60 min) for the animals to reach a glucose level <60 mg/dl. In all cases, glucose nadirs were <60 mg/dl, and C-peptide was not measurable. (△), insulin 1.5 units/ml, $n = 5$; (◇), $ZnCl_2$ 30 μmol/l, $n = 3$; (□), zinc-free insulin 1.5 units/ml, $n = 6$; (■), $ZnCl_2$ 4.5 μmol/l, $n = 6$; and (×), no pancreatic artery infusion.

(1–3). Although cessation of insulin secretion and initiation of glucagon secretion cannot be documented to occur at precisely the same glucose concentration when measuring systemic blood, finer discrimination of bidirectional hormonal regulation would be more evident in islet periportal blood if it could be measured. Our study was designed to assess whether zinc atoms bound to and cosecreted with insulin contribute to this switch-off signal. Our in vivo study on streptozotocin-induced diabetic Wistar rats showed that during hypoglycemia, decrements of both zinc-containing insulin and zinc chloride alone, but not zinc-free insulin, signaled the α-cell to secrete glucagon. In this study, the control experiments were performed in the same animals 2 weeks before the pancreaticoduodenal artery infusions, which allowed us to use a paradigm of paired comparisons for statistical analysis. In previous studies (4), we documented that infusion of saline by this route, followed by switching off the saline infusion, does not correct absent glucagon responses to hypoglycemia in this experimental model. Control studies of glucose uptake in L6 muscle cells and HepG2 liver cells documented the bioactivity of our zinc-free insulin preparation. This indicates that zinc atoms, not insulin molecules, provide the switch-off signal to activate the α-cell during hypoglycemia. Previous work by others suggests multiple potential downstream effects of zinc that could participate in zinc action. For example, zinc has been reported to modulate many cellular functions, including

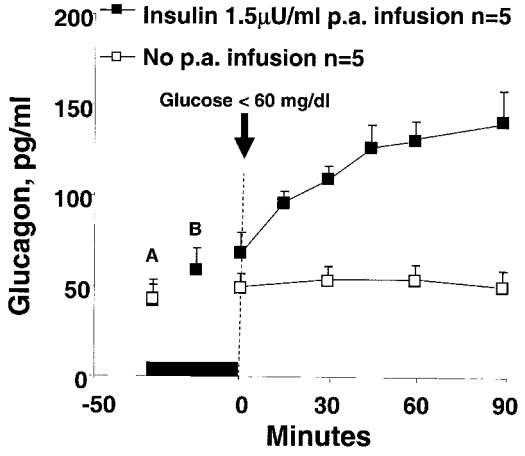

FIG. 2. Levels of glucagon before, during, and after switching off an intrapancreatic artery infusion of regular insulin during hypoglycemia in STZ-induced diabetic rats. See legend for Fig. 1 for infusion protocol. The glucagon response calculated as AUC was significantly greater ($P < 0.05$) than the response in the same animals during insulin-induced hypoglycemia 2 weeks earlier.

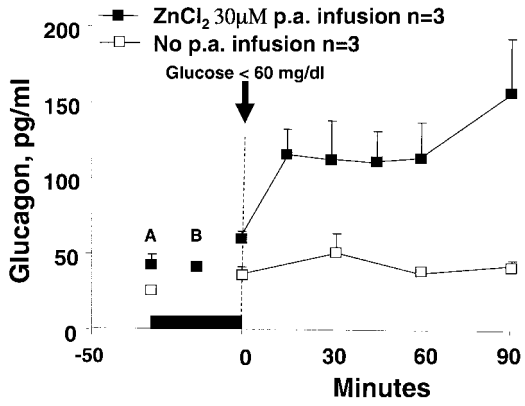

FIG. 3. Levels of glucagon before, during, and after switching off an intrapancreatic artery–zinc chloride (30 μmol/l) infusion during hypoglycemia in STZ-induced diabetic rats. See legend for Fig. 1 for infusion protocol. The glucagon response calculated as AUC was significantly greater ($P < 0.05$) than the response in the same animals during insulin-induced hypoglycemia 2 weeks earlier.

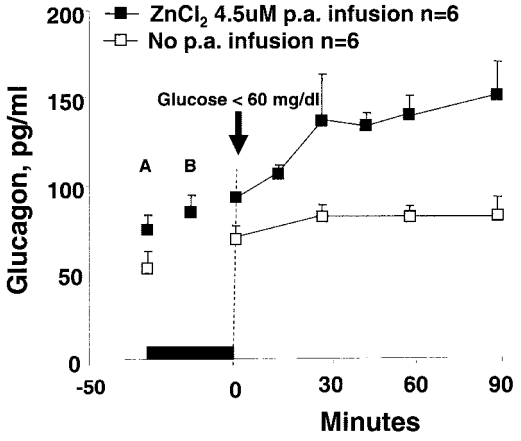

FIG. 4. Levels of glucagon before, during, and after switching off an intrapancreatic artery–zinc chloride (4.5 μmol/l) infusion during hypoglycemia in STZ-induced diabetic rats. See legend for Fig. 1 for infusion protocol. The glucagon response calculated as AUC was significantly greater ($P < 0.02$) than the response in the same animals during insulin-induced hypoglycemia 2 weeks earlier.

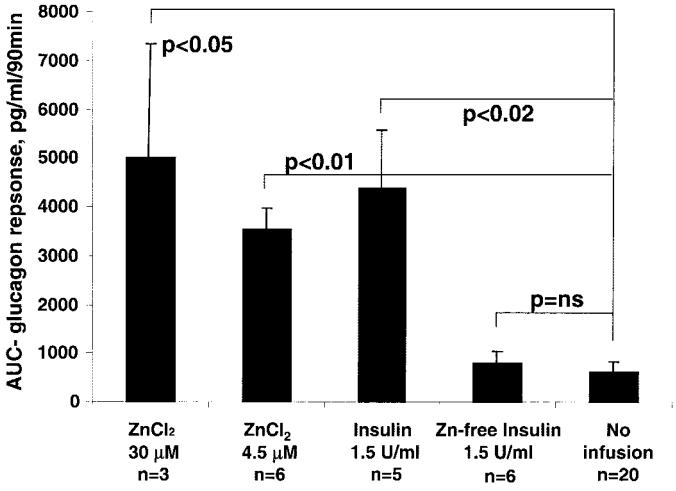

FIG. 6. Comparison of the glucagon responses expressed as AUCs in the four experimental groups versus the control experiment conducted 2 weeks previously.

cell signal recognition, second messenger metabolism, protein kinase, phosphatase activities, and transcription factor activities (11). Long-term exposure of skeletal muscle cells to zinc has been shown to stimulate tyrosine phosphorylation of the insulin receptor and increase glucose uptake (12). We observed only a small increase in zinc-stimulated glucose uptake in the L6 cells, likely due to our short treatment period. Studies of the effect of zinc on hepatoma cells demonstrated an activation of the phosphatidylinositol 3-kinase/Akt pathway (13). While insulin has been shown to stimulate glucose uptake in HepG2 cells (14,15), to our knowledge, our study is the first to demonstrate zinc-stimulated glucose uptake.

The intraislet insulin hypothesis has been debated, in part, because of an early report demonstrating glucagon responses to hypoglycemia in type 1 diabetic subjects (16). However, the diabetic subjects in this study had been diagnosed for only 13–15 months, so it is possible that, although immeasurable in systemic venous blood, residual endogenous insulin secretion was still present in the islet

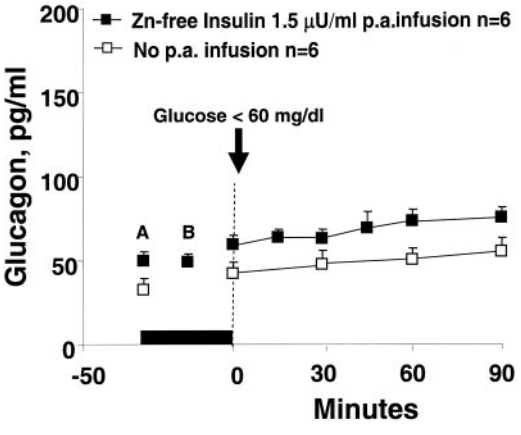

FIG. 5. Levels of glucagon before, during, and after switching off an intrapancreatic artery–zinc-free insulin infusion during hypoglycemia in STZ-induced diabetic rats. See legend for Fig. 1 for infusion protocol. The glucagon response calculated as AUC was not significantly different than the response in the same animals during insulin-induced hypoglycemia 2 weeks earlier.

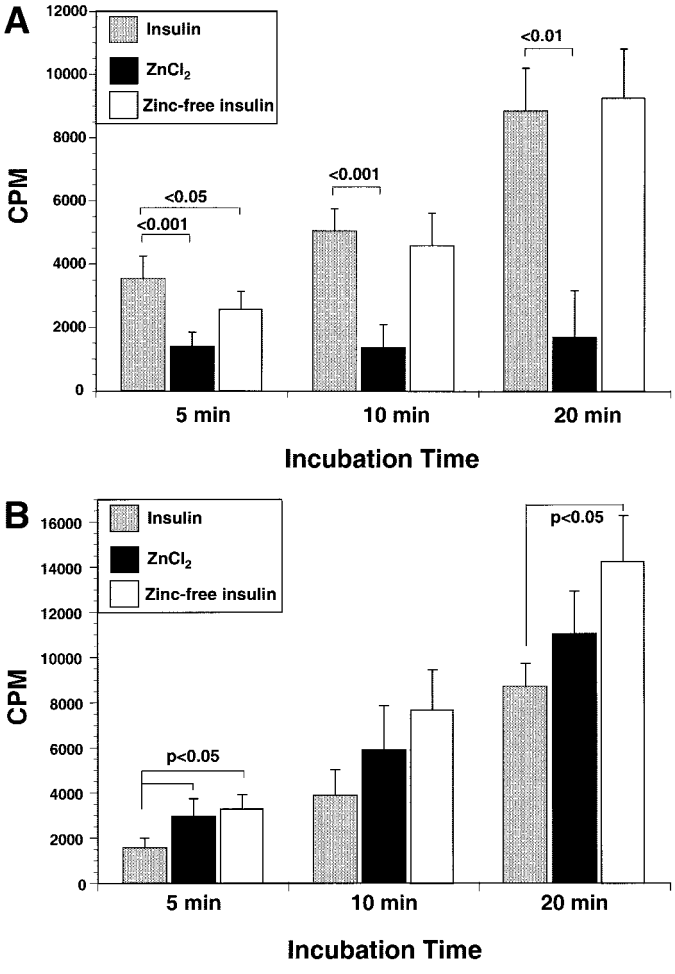

FIG. 7. Demonstration in studies of glucose uptake by L6 muscle cells (*A*) and HepG2 hepatic cell (*B*) lines that the zinc-free preparation used for infusions was as, if not more, biologically active as zinc-containing regular insulin. 1.0 μmol/l concentrations were used for insulin, zinc-free insulin, and zinc chloride. See RESEARCH DESIGN AND METHODS for details.

periportal circulation. More recent support for the intraislet hypothesis was provided by a report that glucose-regulated glucagon secretion requires the presence of insulin receptor expression (17). The hypothesis that zinc might be regulating glucagon secretion has been suggested most prominently by Wollheim's group (7,9) reporting that glucagon secretion is sensitive to suppression by zinc (EC_{50} = 2.7 μmol/l for $ZnCl_2$, following earlier reports that the SUR1/KIR6.2-type K_{ATP} channels present in islet α- and β-cells are activated by zinc) (6,8). Zinc activation of K_{ATP} channels will hyperpolarize α-cells and thus decrease the influx of cytosolic Ca^{2+} necessary for glucagon secretion. Alternatively, it has been suggested by Gopel et al. (18) that closure of K_{ATP} channels could inactivate the Na^+ current, leading to hyperpolarization and cessation of Ca^{2+} influx. A blunted glucagon response to hypoglycemia in Sur1-knockout mice lacking neuroendocrine-type K_{ATP} channels supports this concept (19). A report questioning a regulatory role of zinc for glucagon secretion in mice has recently appeared. This study observed that $ZnCl_2$ did not suppress glucagon secretion from mouse islets (20), suggesting a significant species difference (mice vs. rats) as one explanation for these negative findings. In this work, the authors performed experiments using an α-cell line to demonstrate decreased glucagon secretion when it was exposed to 10 rather than 0 mmol/l glucose, a blocking effect of Wortmanin on insulin-mediated suppression of glucagon secreton, and the inability of Wortmanin to block glucose suppression of glucagon secretion.

None of these previous studies examined hypoglycemia as the initiator of glucagon secretion or the role that insulin versus zinc might play as a regulator of glucagon secretion. Since the preparation of zinc-free insulin we used caused systemic hypoglycemia when injected into the jugular vein and was active in stimulating glucose uptake in two cell lines, zinc is clearly not required for insulin action in these situations. Therefore, we reason that the zinc signaling effect on the α-cell we observed may be specific to the setting of hypoglycemia. (see NOTE ADDED IN PROOF) The fact that zinc, independent of insulin, can have insulin-like effects is well appreciated (21). Furthermore, other endogenous substances, such as intra-α-cell L-glutamate directly or via stimulation of γ-aminobutyric acid secretion from β-cells, may also negatively regulate glucagon secretion (22).

In conclusion, we propose that during hypoglycemia the principal signal that initiates glucagon secretion is the detection by α-cells of a sudden decrease in zinc paralleling the fall in insulin in the islet periportal circulation and that this drop in concentration of zinc atoms, not insulin molecules themselves, closes α-cell K_{ATP} channels, promotes entry of calcium, and stimulates glucagon secretion.

NOTE ADDED IN PROOF

One of the reviewers of this manuscript requested that we speculate what the pancreaticoduodenal artery blood zinc concentration might be during our infusions of zinc choloride. If one assumes that blood volume of a rat is 24 cc, that blood zinc concentration is normally 10 μmol/l, that circulation time is 3.3 s, and that 1% of total blood volume or 0.24 cc passes through the artery every 10 s, then ~4.8 cc passes through every minute at a concentration of 10 μmol/l zinc. Since the zinc chlorida infusion rate is 50 μmol/l or 0.05 cc/min, using a concentration of 4 mmol/l would provide 1:100 (by volume) enrichment by 4,000 μmol/l for blood containing 10 μmol/l zinc and flowing at 4.8 cc/min. This would result in a Δ of 10–40 μmol/l or a fourfold step up in zinc concentration reaching the α-cell. Of course, none of these calculations take into account the probable influence of percentage of protein binding of zinc in blood, which begs the question regarding the concentration of free zinc ions. These issues will no doubt be more accurately approached using an in vitro methodology, such as islet perifusion and measurements of bound and unbound zinc in the perifusate.

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