EXENDIN-(9-39) CORRECTS FASTING HYPOGLYCEMIA IN SUR-1\(^{-/-}\) MICE BY LOWERING cAMP IN PANCREATIC \(\beta\)-CELLS AND INHIBITING INSULIN SECRETION

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Running Title: Effect of Exendin-(9-39) in SUR-1\(^{-/-}\) mice

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Summary

Congenital hyperinsulinism is a disorder of pancreatic \(\beta\)-cell function characterized by failure to suppress insulin secretion in the setting of hypoglycemia, resulting in brain damage or death if untreated. Loss-of-function mutations in the KATP channel (composed of two subunits: Kir6.2 and SUR-1) are responsible for the most common and severe form of congenital hyperinsulinism. Most patients are unresponsive to available medical therapy and require palliative pancreatectomy. Similar to the human condition, the SUR-1\(^{-/-}\) mouse is hypoglycemic when fasted and hyperglycemic when glucose loaded. We have previously reported that the glucagon-like peptide-1 receptor antagonist exendin-(9-39) raises fasting blood glucose in normal mice. Here we examine the effect of exendin-(9-39) on fasting blood glucose in SUR-1\(^{-/-}\) mice. Mice were randomized to receive exendin-(9-39) or vehicle. Fasting blood glucose levels in SUR-1\(^{-/-}\) mice treated with exendin-(9-39) were significantly higher than in vehicle treated mice and not different from wild-type littermates. Exendin-(9-39) did not further worsen glucose tolerance and had no effect on body weight and insulin sensitivity. Isolated islet perifusion studies demonstrated that exendin-(9-39) blocked amino acid-stimulated insulin secretion, which is abnormally increased in SUR-1\(^{-/-}\) islets. Furthermore, cAMP content in SUR-1\(^{-/-}\) islets was reduced by exendin-(9-39) both basally and when stimulated by amino acids, whereas cytosolic calcium levels were not affected. These findings suggest that cAMP plays a key role in KATP-independent insulin secretion and that the GLP-1 receptor is constitutively active in SUR-1\(^{-/-}\) \(\beta\)-cells. Our findings indicate that exendin-(9-39) normalizes fasting hypoglycemia in SUR-1\(^{-/-}\) mice via a direct effect on insulin secretion, thereby raising exendin-(9-39) as a potential therapeutic agent for K\(_{\text{ATP}}\) hyperinsulinism.

Introduction

Congenital hyperinsulinism (CHI) is a genetic disorder of pancreatic \(\beta\)-cell function characterized by failure to suppress insulin secretion in the setting of hypoglycemia, resulting in brain damage or death if inadequately treated. Loss-of-function mutations in the K\(_{\text{ATP}}\)-sensitive channel (composed of two subunits: Kir6.2 and SUR-1) are responsible for the most common and severe form of hyperinsulinism (K\(_{\text{ATP(HI)}}\)). K\(_{\text{ATP}}\)-sensitive channels couple the metabolic state of the \(\beta\)-cell to membrane potential by sensing changes in
intracellular ATP concentration. In pancreatic β-cells, closure of the channel in response to elevation of the ATP/ADP ratio following stimulation with glucose leads to depolarization of the membrane and activation of voltage-dependent calcium channels with resultant exocytosis of insulin-containing granules (1). Thus, K<sub>ATP</sub>-sensitive channels play a critical role in the triggering pathway of glucose-stimulated insulin secretion (2). In contrast, the amplifying pathway of insulin release operates independently of K<sub>ATP</sub>-sensitive channels and functions to augment fuel-stimulated insulin secretion (3). Electrophysiological studies of islets from infants with K<sub>ATP</sub>HI show reduction of K<sub>ATP</sub> channel activity and spontaneously active voltage-dependent Ca<sup>2+</sup> channels (reviewed in (4)). Diazoxide, the mainstay of medical therapy for hyperinsulinism, suppresses insulin by promoting the opening of the β-cell K<sub>ATP</sub> channel and is ineffective in patients with K<sub>ATP</sub>HI. Thus, most of these patients require surgical palliation by near-total pancreatectomy, which is not curative but carries a high risk of either persistent hypoglycemia or insulin-requiring diabetes (5).

An animal model harboring a targeted inactivation of the SUR-1 gene (SUR-1<sup>−/−</sup> mouse) reproduces the key pathophysiological features of K<sub>ATP</sub>HI. SUR-1<sup>−/−</sup> mice are both significantly more hypoglycemic when fasted and significantly more hyperglycemic when glucose-loaded compared to control animals (6). Interestingly, SUR-1<sup>−/−</sup> mice secrete a nearly normal amount of insulin in response to a meal despite markedly impaired glucose-stimulated insulin secretion (7). Isolated SUR-1<sup>−/−</sup> islets exhibit all the features expected to result from nonfunctional K<sub>ATP</sub> channels, including β-cell depolarization (6) and elevation of intracellular calcium (7-9). Furthermore, baseline insulin release in cultured SUR-1<sup>−/−</sup> islets is higher compared to control islets (8,9). Segers et al. (6) demonstrated a lack of first phase insulin secretion in response to glucose and attenuated second phase insulin release in SUR-1<sup>−/−</sup> islets. The authors concluded that in the absence of K<sub>ATP</sub> channels, insulin release is regulated by a slow, K<sub>ATP</sub>-independent glucose stimulated mechanism. In contrast to the defect in glucose-stimulated insulin release, isolated SUR-1<sup>−/−</sup> islets are hypersensitive to amino acids. After stimulation with an amino acid mixture, insulin release increases by 3-fold in SUR-1<sup>−/−</sup> islets, while normal islets do not respond to amino acids in the absence of a concomitant glucose stimulus. Glutamine plays a prominent role in mediating amino acid stimulation of insulin release in SUR-1<sup>−/−</sup> islets, and this effect requires functional calcium channels as well as β-cell depolarization (8).

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by the intestinal L-cells in response to ingested nutrients that acts on β-cells to augment insulin release in response to glucose (10). GLP-1 stimulates insulin secretion by binding to a guanine nucleotide binding protein-coupled receptor (GPCR) (11) resulting in the activation of adenylate cyclase and generation of 3'5' cyclic adenosine monophosphate (cAMP) (12). GLP-1 and agents that increase cAMP levels stimulate insulin secretion by both PKA-dependent and -independent mechanisms (13). Studies of the GLP-1 response in SUR-1<sup>−/−</sup> islets have provided mixed results. Nakazaki et al (14) reported that despite a normal cAMP elevation to GLP-1, stimulation with GLP-1 did not affect insulin release in SUR-1<sup>−/−</sup> islets. In contrast, Eliasson et al (15) demonstrated that GLP-1 stimulates insulin secretion in SUR-1<sup>−/−</sup> islets, although the magnitude of the response was reduced to ~50% of wild-type islets. The latter study also found an impairment of the PKA-independent component of exocytosis in SUR-1<sup>−/−</sup> islets (15). Further, Doliba et al (16) demonstrated that GLP-1 (as well as acetylcholine) restores glucose responsive insulin secretion in SUR-1<sup>−/−</sup> islets, again supporting the concept that elevation of intracellular cAMP levels further augments insulin secretion in the face of persistently high intracellular calcium levels.

Exendin-(9-39), a derivative of the nonmammalian peptide exendin-4, acts as a specific and competitive antagonist of the GLP-1 receptor and impairs glucose tolerance in humans (17) as well as in a variety of animal models (18-20). Studies in murine β-cells have shown that exendin-(9-39) decreases basal cAMP levels, acting as an inverse agonist of the GLP-1 receptor (21). We have previously shown that exendin-(9-39) results in a persistent elevation of fasting
blood glucose levels in wild-type BALB-c mice (22). Based on this observation and on the evidence for GLP-1 responsiveness of SUR-1 -/- islets, we hypothesized that exendin-(9-39) would increase fasting blood glucose in SUR-1 -/- mice. Here we show that antagonism of the GLP-1 receptor by exendin-(9-39) corrects fasting hypoglycemia in SUR-1 -/- mice via a direct effect on insulin secretion. Further, exendin-(9-39) decreases basal and stimulated cAMP levels significantly in isolated SUR-1 -/- islets, mirroring its impact on the insulin secretory response of these islets. Taken together, these data identify exendin-(9-39) as a potential therapeutic agent in human patients with congenital hyperinsulinism.

Materials and Methods

Animals
SUR-1 -/- mice were kindly provided by Dr. Mark A. Magnuson. The generation and genotyping of SUR-1 -/- mice were previously described (7). Mice are maintained in a C57Bl/6 genetic background. Twelve to eighteen week SUR-1 -/- and wild-type littermate control mice were used in all experiments. Mice were maintained on a 12:12-h light-dark cycle and were fed a standard rodent chow diet. All procedures were approved and carried out according to the University of Pennsylvania Institutional Animal Care and Use Committee guidelines.

Exendin-(9-39) administration
Alzet mini-osmotic pumps (model 2002; Alza, Palo Alto, CA) were implanted subcutaneously to deliver exendin-(9-39) (Bachem Bioscience, King of Prussia, PA) at a rate of 150 pmol/kg/min or vehicle (0.9% NaCl/1% BSA) for 2 weeks.

Glucose homeostasis
For determination of fasting blood glucose levels mice were fasted for 12-16 hours. Oral glucose tolerance testing was carried after a 12-16 hour fast by administering 2g/kg of dextrose by oral gavage (feeding needles; Popper and Sons, Inc., Hyde Park, NY). For insulin tolerance testing mice received 0.5 units/kg of insulin intraperitoneally after a 4 hour fast. Blood glucose levels were measured using a hand-held glucose meter (FreeStyle; TheraSense, Alameda, CA). Insulin and glucagon were measured by ELISA (Mouse Endocrine Immunoassay Panel; Linco Research, Inc., St. Charles, MO).

Islet Studies
Islets were isolated by collagenase digestion and cultured for 3 days in RPMI 1640 medium containing 10 mM glucose. The culture medium was supplemented with 10% fetal bovine serum, 2mM glutamine, 100 units/mL penicillin, and 50 µg/mL streptomycin. Islets were incubated at 37°C in a 5% CO2, 95% air-humidified incubator. Batches of 10 cultured mouse islets were loaded onto a nylon filter in a chamber and perifused with Krebs-Ringer bicarbonate buffer (115 mM NaCl, 24 mM NaHCO3, 5 mM KCl, 1 mM MgCl2, 2.5 mM CaCl2, 10mM HEPES, pH 7.4) with 0.25% bovine serum albumin at a flow rate of 2 mL/min. Perifusate solutions were gassed with 95% O2, 5% CO2 and maintained at 37°C. Islets were stimulated with a ramp of amino acids. The mixture of 19 amino acids when used at a maximum concentration of 12 mM (about 3 times physiological concentration) had the following composition (in mM): glutamine 2, alanine 1.25, arginine 0.53, aspartate 0.11, citrulline 0.27, glutamate 0.35, glycine 0.85, histidine 0.22, isoleucine 0.27, leucine 0.46, lysine 1.06, methionine 0.14, ornithine 0.20, phenylalanine 0.23, proline 1, serine 1.62, threonine 0.77, tryptophan 0.21, valine 0.57. Samples were collected every minute for insulin assays. Insulin was measured by radioimmunoassay (Linco Research Inc., St. Charles, MO).

cAMP content determination
Islets were isolated as above, hand-picked and cultured for three days. Cultured islets were preincubated in glucose free Krebs-Ringer bicarbonate buffer for 60 min, 100 nM exendin-(9-39) was added 30 min into the preincubation period. Then, islets were exposed to different treatments for an additional 30 min in the presence of 0.1mM isobutyl-methylxanthine (IBMX). After incubation, islets were washed 2 times by cold glucose-free Hank's buffer. cAMP was measured in islet lysates by ELISA (GE/Amersham Biosciences, Piscataway, NJ).
Cytosolic Free Ca\textsuperscript{2+} Measurements

Mouse islets were isolated and cultured on poly-L-lysine coated glass coverslips under the same conditions as described above. The perfusion procedure and cytosolic-free Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) measurement were described previously (23). In brief, the coverslip with attached islets was incubated with 15 \( \mu \)M Fura-2 acetoxymethylester (Molecular Probes, Eugene, OR) in Krebs-Ringer bicarbonate buffer with 5 mM glucose for 35 min at 37 °C. Islets were then perifused with Krebs-Ringer bicarbonate buffer with 0.25 % bovine serum albumin at 37 °C at a flow rate of 2mL/min, while various agents were applied. [Ca\textsuperscript{2+}]\textsubscript{i} was measured with a dual wavelength fluorescence microscope as previously described.

Statistical evaluation

Data presented are mean ± SEM and compared using student’s t test. For glucose and insulin tolerance testing, values were compared by repeated-measures ANOVA. Differences were considered significant at p<0.05.

Results

Twelve-18 week old male \textit{SUR-1}\textsuperscript{-/-} and wild-type littermates underwent a baseline evaluation including fasting blood glucose measurements and oral glucose tolerance testing, followed by randomization to treatment with exendin-(9-39) (150 pmol/kg/min) or vehicle (0.9% NaCl, 1% BSA). Fasting glucose levels were determined after an overnight fast on days 3 and 7 of the infusion. In addition, oral glucose tolerance and insulin sensitivity were evaluated during treatment.

As previously reported (7), fasting blood glucose levels were significantly lower in \textit{SUR-1}\textsuperscript{-/-} mice compared to wild-type littermates (59.4 ± 1.5 mg/dL vs. 75 ± 1.8 mg/dL, \( p=0.00000003 \)) (Figure 1A), while body weight was not different (Figure 1B). After an oral load of glucose, \textit{SUR-1}\textsuperscript{-/-} mice were glucose intolerant when compared to littermate wild-type controls (\( p<0.0001 \), repeated measures ANOVA) (Figure 1C). The \textit{SUR-1}\textsuperscript{-/-} mice have a significant impairment of insulin secretion in response to an oral glucose load (wild-type vs. \textit{SUR-1}\textsuperscript{-/-}: \( p=0.02 \), repeated measures ANOVA) (Figure 1D).

Exendin-(9-39) was administered via an Azlet miniosmotic subcutaneous pump at a continuous infusion rate of 150 pmol/kg/min for 2 weeks. This dose was chosen based on results of a pilot study evaluating different doses previously shown to have an effect in normal humans and mice (17,22,24). On day 7, fasting blood glucose was significantly lower in vehicle-treated \textit{SUR-1}\textsuperscript{-/-} mice compared to vehicle-treated wild-type littermates (\( p=0.000002 \)) (Figure 2). Treatment with exendin-(9-39) significantly raised fasting blood glucose levels in \textit{SUR-1}\textsuperscript{-/-} mice compared to vehicle-treated \textit{SUR-1}\textsuperscript{-/-} mice (82.2 ± 6.3 mg/dL vs. 63.2 ± 4.9 mg/dL, \( p=0.03 \), on day 3; 82 ± 4.7 mg/dL vs. 56.4 ± 4.3 mg/dL, \( p=0.0006 \), on day 7). Fasting blood glucose levels were not different in exendin-(9-39)-treated wild-type mice compared to vehicle-treated wild-type controls, in contrast to our previous observations in wild-type BALB/c mice. Exendin-(9-39) did not impact weight gain in \textit{SUR-1}\textsuperscript{-/-} nor wild-type littermate controls (data not shown).

During treatment, fasting insulin and glucagon levels were not significantly different among the treatment groups (Figure 3A); however in the setting of lower fasting blood glucose levels, insulin levels were inappropriately elevated in vehicle treated \textit{SUR-1}\textsuperscript{-/-} mice and glucagon levels failed to rise as expected in response to the hypoglycemia. Insulin/glucose ratio is increased in \textit{SUR-1}\textsuperscript{-/-} mice compared to wild-type littermate controls (Figure 3B) (WT vs. \textit{SUR-1}\textsuperscript{-/-}: \( p=0.04 \)) and is normalized by exendin-(9-39) treatment (WT vs. \textit{SUR-1}\textsuperscript{-/-}Ex-(9-39): \( p=0.32 \)), suggestive of a direct islet effect of exendin-(9-39) on insulin secretion.

Despite the marked effect on fasting blood glucose levels, treatment with exendin-(9-39) did not significantly impair glucose tolerance in \textit{SUR-1}\textsuperscript{-/-} mice, except for a delay in the return to baseline blood glucose levels at the 120 minute time point. Similarly, there was no effect on glucose tolerance in wild-type littermates during
treatment with exendin-(9-39) (Figure 4A). In agreement with these results, glucose-stimulated insulin secretion was not affected by exendin-(9-39) in wild-type mice and the already impaired insulin response to the oral glucose load characteristic of the SUR-1–/– mice was not further impaired by treatment (Figure 4B).

To determine the mechanism of action for the effect of exendin-(9-39) on fasting blood glucose levels we assessed insulin sensitivity by an insulin tolerance test. Insulin sensitivity was not different between SUR-1–/– and wild-type littermates. Further, Exendin-(9-39) did not impact peripheral insulin sensitivity in any of the treatment groups (Figure 4C).

Given the observed in vivo effects on insulin secretion but not insulin sensitivity, we next determined whether exendin-(9-39) exerts a direct effect on SUR-1–/– islet function. Since SUR-1–/– islets do not respond to glucose under our experimental conditions (8), we chose to examine the effect of exendin-(9-39) on the abnormal response of SUR-1–/– islets to fuel-induced insulin secretion (specifically, the hyper-responsiveness to amino acids). Isolated islets were perifused with a mixture of amino acids. As previously reported (8), SUR-1–/– islets abnormally released insulin in response to ramp stimulation by a physiologic mixture of 19 amino acids (using an increment of 0.04 mM/min for glutamine and 0.2 mM/min for the other amino acids). This response to amino acids was blocked by exendin-(9-39) (Figure 5A). The insulin response to KCl was similar in the presence and absence of exendin-(9-39).

To examine the effect of exendin-(9-39) in wild-type islets we incubated islets with 10 mM glucose and then introduced the ramp of amino acids. Li et al. (8) previously demonstrated that in the presence of 10 mM glucose, a ramp of amino acids stimulates insulin secretion in a concentration dependent manner. Here we demonstrate that, as in SUR-1–/– islets, exendin-(9-39) specifically inhibits the response to amino acids in wild-type islets (Figure 5B). In contrast, the acute response to glucose (10 mM) was not impaired by exendin-(9-39).

The effect of exendin-(9-39) on cAMP was determined in static incubations of isolated islets. In the absence of exogenous GLP-1, exendin-(9-39) significantly decreased basal intracellular cAMP in SUR-1–/– islets (40 ± 4 vs. 21 ± 2 pmol/100 islets, p<0.05) (Table 1). Amino acids significantly increased cAMP levels in SUR-1–/– islets compared to baseline (73 ± 13 vs. 40 ± 4 pmol/100 islets, p<0.05). The amino acid-stimulated increase in cAMP was significantly reduced by exendin-(9-39) (73 ± 13 vs. 24 ± 5 pmol/100 islets, p<0.05). In these static incubations, the effect of exendin-(9-39) on cAMP levels mirrored the effect on insulin secretion, suggesting that exendin-(9-39) effects on insulin secretion in SUR-1–/– islets are mediated by changes in cAMP. Baseline insulin secretion was significantly reduced by exendin-(9-39) (221 ± 22 vs. 126 ± 17 ng/100 islets/30 min, p<0.05). As seen in the perifusion studies, amino acids significantly increased insulin secretion in SUR-1–/– islets (221 ± 22 vs. 360 ± 32 ng/100 islets/30 min, p<0.01), and exendin-(9-39) significantly reduced amino acid-stimulated insulin secretion (360 ± 32 vs. 190 ± 35 ng/100 islets/30 min, p<0.01).

Under glucose-free conditions, cAMP levels were higher in wild-type islets compared to SUR-1–/– islets (83 ± 3 vs. 40 ± 4 pmol/100 islets, p<0.01). This difference may be explained by the high insulin levels in SUR-1–/– islets. In support, previous reports have shown that insulin can reduce cellular cAMP by activating phosphodiesterase to induce cAMP degradation (25-28).

In wild-type islets, exendin-(9-39) significantly decreased basal intracellular cAMP in (83 ± 3 vs. 47 ± 6 pmol/100 islets, p<0.01) (Table 2). Cyclic AMP was not significantly increased when glucose concentration was increased from 0 to 10 mM. Amino acids significantly increased cAMP levels in wild-type islets in the presence of 10 mM of glucose compared to baseline (95 ± 6 vs. 62 ± 4 pmol/100 islets, p<0.05). The amino acid-stimulated increase in cAMP was significantly reduced by exendin-(9-39) (95 ± 6 vs. 42 ± 2 pmol/100 islets, p<0.01).
In wild-type islets, baseline insulin secretion (in the absence of any stimuli) was not affected by exendin-(9-39). Insulin secretion as expected was significantly stimulated by 10 mM of glucose (17 ± 4 vs. 153 ± 10 ng/100 islets/30 min, p<0.01). In contrast to the lack of effect on glucose-stimulated insulin secretion in the perifusion studies, exendin-(9-39) significantly reduced glucose-stimulated insulin secretion in these static incubations (153 ± 10 vs. 72 ± 11 ng/100 islets/30 min, p<0.01). Flamez et al (29) have reported variable effects of exendin-(9-39) depending on the length of incubation, this may explain the different effect in our perifusion and static incubation experiments. As seen in the perifusion studies, amino acids significantly increased insulin secretion in wild-type islets in the presence of glucose (463 ± 27 vs. 153 ± 10 ng/100 islets/30 min, p<0.01), and exendin-(9-39) significantly reduced amino acid-stimulated insulin secretion (463 ± 27 vs. 153 ± 18 ng/100 islets/30 min, p<0.01).

Finally, we studied the effect of exendin-(9-39) on the characteristically elevated intracellular calcium concentration of SUR-1−/− islets (8). Exendin-(9-39) did not affect basal intracellular calcium (Figure 6). As previously reported, amino acids caused a transient further rise in intracellular calcium. Exendin-(9-39) had no effect on the amino acid-stimulated rise in intracellular calcium, indicating that its effect on insulin secretion is distal to the effect of calcium.

Discussion

Congenital hyperinsulinism due to mutations in the K_{ATP} channel is a devastating disease that is generally unresponsive to available medical therapies. We previously reported that a continuous infusion of exendin-(9-39) elevated fasting blood glucose levels in BALB/c mice (22), an effect that has also been observed in baboons (19) and healthy human subjects (24). Although SUR-1−/− mice are relatively normoglycemic in the fed state (6,7), they develop hypoglycemia with fasting, which allowed us to test the potential for exendin-(9-39) to normalize fasting blood glucose in the absence of functional K_{ATP} channels.

Our studies confirmed previous reports highlighting the phenotype of the SUR-1−/− mice (6,7). Compared to wild-type littermates, SUR-1−/− mice are more hypoglycemic when fasted and more hyperglycemic when glucose loaded, and the glucose intolerance is the result of impaired insulin secretion. The islet counter-regulatory response to hypoglycemia is also impaired in the SUR-1−/− mice, since in the fasting state, when blood glucose is significantly lower than in wild-type littermates, insulin and glucagon levels are not different compared with normoglycemic wild-type littermates. These findings are in agreement with those of Shiota et al (30) who reported an impaired glucagon secretory response in SUR-1−/− mice.

Administered as a continuous infusion, exendin-(9-39) significantly raised fasting blood glucose levels in SUR-1−/− mice without significantly impacting weight gain, glucose tolerance or insulin sensitivity. However, we cannot entirely rule out a contribution by changes in hepatic insulin sensitivity, which could have been missed on the insulin tolerance test. The elevated fasting insulin/glucose ratio in SUR-1−/− mice was decreased by exendin-(9-39). We did not observe an effect of exendin-(9-39) on glucagon levels in the fasting state. These findings suggest that the effect of exendin-(9-39) on fasting blood glucose levels is mediated by the β-cell GLP-1 receptor with no significant impact on other peripheral or central GLP-1 receptor-mediated actions at the dose studied.

The striking in vivo impact of exendin-(9-39) on fasting blood glucose in the SUR-1−/− mice prompted us to explore its effects on the nutrient-induced insulin secretory response of SUR-1−/− islets. Because of the clinical observation that protein ingestion provokes hypoglycemia in patients with K_{ATP} hyperinsulinism (31), and our observation that SUR-1−/− islets hyper-respond to amino acids (8) we evaluated the impact of exendin-(9-39) on amino acid-stimulated insulin secretion. In islet perifusion studies, exendin-(9-39) suppressed amino acid-stimulated insulin secretion in both SUR-1−/− and wild-type islets Although the
mechanism by which amino acids, specifically glutamine, stimulates insulin secretion is not well understood (8) our findings suggest that cAMP plays a central role in amino acid-stimulated insulin secretion. Further, exendin-(9-39) decreased basal and amino-acid stimulated insulin secretion and intracellular cAMP accumulation in static incubation experiments. Thus, exendin-(9-39) corrects the abnormal pattern of insulin secretion responsible for hypoglycemia in the absence of K<sub>ATP</sub> channels: elevated basal and amino acid-stimulated insulin secretion.

The results of isolated islet experiments, conducted in the absence of exogenous GLP-1 receptor ligand, suggest that the GLP-1 receptor is constitutively active in SUR-1<sup>+</sup> islets and they support previous reports implicating exendin-(9-39) as an inverse agonist of the GLP-1 receptor (21,29). Alternatively, this effect might be explained by antagonism of local intra-islet GLP-1 (32), or by competition between exendin-(9-39) and glucagon for binding to the GLP-1 receptor on β-cells (33).

Nakazaki et al (14), reported a loss of cAMP-induced potentiation of insulin secretion in SUR-1<sup>−/−</sup> islets. In their studies, GLP-1-induced elevation of cAMP was not mirrored by changes in insulin secretion in mutant islets. Interestingly and in contrast to these observations, the changes we observed in cAMP levels were mirrored by changes in insulin secretion. This discrepancy may be attributable to methodologic differences. In our studies, islets were cultured for three days prior to perfusion or static incubation experiments. We believe that this pre-culture recovery period is essential to restore a normal secretory response after the stress of islet isolation.

Cyclic AMP has been previously recognized as a critical physiological potentiator of insulin secretion (34). Our studies demonstrate the central role of cAMP in the K<sub>ATP</sub> channel-independent pathway regulating insulin secretion. Cyclic AMP stimulates exocytosis by PKA-dependent pathways, through phosphorylation of downstream targets including the K<sub>ATP</sub> channel, and by PKA-independent mechanisms, through the activation of guanine nucleotide exchange factors (GEFs) such as cAMP-GEFII (also known as Epac2) (13,35). The PKA-independent pathway is critical in the potentiation of insulin secretion by the incretin hormones GLP-1 and GIP (36). Furthermore, Epac2 plays an essential role in the first phase of insulin granule exocytosis potentiated by cAMP (37). Cyclic AMP promotes insulin granule exocytosis by increasing the size of the readily releasable pool and by accelerating the refilling of the readily releasable pool (12,13,15,38). In pancreatic islets, the effect of Epac2 on insulin secretion depends on cytosolic calcium as well as cAMP (36), and it has been postulated that cAMP sensitizes the exocytotic machinery to calcium (39). Thus, we speculate that the inhibition of insulin secretion in SUR-1<sup>−/−</sup> islets by exendin-(9-39) is mediated by the effect of cAMP on a late calcium-dependent step in the exocytotic pathway (Figure 7).

In summary, we have shown that exendin-(9-39) significantly raises fasting blood glucose levels in SUR-1<sup>−/−</sup> mice through a direct effect on insulin secretion that appears to be mediated at least in part by changes in intracellular cAMP accumulation. These findings have significant translational application, given the lack of effective medical therapies for children with congenital hyperinsulinism due to K<sub>ATP</sub> mutations. The use of GLP-1 receptor antagonists to control hypoglycemia in congenital hyperinsulinism could have a beneficial effect on morbidity and long-term outcome in this patient population.
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References

Figure Legends

Figure 1. **Fasting hypoglycemia and impaired glucose tolerance in SUR-1<sup>-/-</sup> mice.** (A) Fasting blood glucose levels (in mg/dL) in SUR-1<sup>-/-</sup> mice (n=27) and wild-type littermate controls (n=30), p=0.00000003. (B) Body weight (in g) in SUR-1<sup>-/-</sup> mice (n=27) and wild-type littermate controls (n=30). (C) Oral glucose tolerance (2g/kg) in SUR-1<sup>-/-</sup> mice (n=23) (solid line and circles) and wild-type littermate controls (n=25) (dashed line and open squares), p<0.0001, repeated measures ANOVA. (D) Insulin secretion in response to an oral glucose load (2g/kg) in SUR-1<sup>-/-</sup> mice (n=8) (solid line and circles) compared to wild-type littermate controls (n=9) (dashed line and open squares), p=0.02, repeated measures ANOVA.

Figure 2. **Exendin-(9-39) normalized fasting blood glucose levels in SUR-1<sup>-/-</sup> mice.** Blood glucose levels were determined after a 12-16 hour fast on day 7. Vehicle-treated wild-type littermates (n=13) (white bar); exendin-(9-39) treated wild-type littermates (n=10) (hatched bar); vehicle-treated SUR-1<sup>-/-</sup> mice (n=11) (black bar); exendin-(9-39) treated SUR-1<sup>-/-</sup> mice (n=11) (gray bar).

Figure 3. **Fasting hormonal profile.** (A) Fasting glucagon and insulin levels in vehicle-treated wild-type (n=15) (white bar), exendin-(9-39)-treated wild-type (n=14) (hatched bar), vehicle-treated SUR-1<sup>-/-</sup> (n=13) (black bar), and exendin-(9-39)-treated SUR-1<sup>-/-</sup> (n=14) (gray bar). (B) Insulin to glucose ratio in vehicle-treated wild-type (n=9) (white bar), exendin-(9-39)-treated wild-type (n=10) (hatched bar), vehicle-treated SUR-1<sup>-/-</sup> (n=9) (black bar), and exendin-(9-39)-treated SUR-1<sup>-/-</sup> (n=10) (gray bar).

Figure 4. **Exendin-(9-39) did not influence glucose tolerance or insulin sensitivity.** (A) Blood glucose levels in response to an oral glucose load in vehicle-treated wild-type littermates (n=10) (dashed line/open squares), exendin-(9-39)-treated wild-type (n=8) (solid line/solid squares), vehicle-treated SUR-1<sup>-/-</sup> mice
Vehicle-treated wild-type vs. vehicle-treated $SUR^{-1/2^-}$, $p=0.001$, repeated measures ANOVA; vehicle-treated $SUR^{-1/2^-}$ vs. exendin-(9-39)-treated $SUR^{-1/2^-}$, $p=0.02$ at 120 min. (B) Insulin levels in response to an oral glucose load in vehicle-treated wild-type littermates (n=10) (dashed line/open squares), exendin-(9-39)-treated wild-type (n=8) (solid line/solid squares), vehicle-treated $SUR^{-1/2^-}$ mice (n=9) (dashed line/open circles), and exendin-(9-39)-treated $SUR^{-1/2^-}$ (n=9) (solid line/solid circles). (C) Blood glucose change (expressed as the % from baseline) in response to an intraperitoneal injection of insulin in vehicle-treated wild-type mice (n=15) (dashed line/open squares), exendin-(9-39)-treated wild-type mice (n=14) (solid line/solid squares), vehicle-treated $SUR^{-1/2^-}$ mice (n=13) (dashed line/open circles), and exendin-(9-39)-treated $SUR^{-1/2^-}$ mice (n=14) (solid line/solid circles).

**Figure 5. Effect of exendin-(9-39) on fuel responsiveness of $SUR^{-1/2^-}$ and wild-type islets.** Isolated islets from $SUR^{-1/2^-}$ mice and wild-type littermates were cultured for 3 days in RPMI 1640 medium containing 10 mM glucose. (A) $SUR^{-1/2^-}$ islets were perifused with a ramp of a physiologic mixture of amino acids (0-12 mM) in the presence (open circles) or absence (black circles) of exendin-(9-39) at a concentration of 100 nM. Finally, 30 mM KCl were applied. (B) Wild-type islets were perifused with (open diamonds) or without (black diamonds) 100 nM exendin-(9-39). Perifused islets were then exposed to 10 mM glucose at 30 min, followed 20 min later by ramp of a physiologic mixture of amino acids (0-12 mM). Finally, 30 mM KCl were applied. Results are presented as means ± S.E. for 100 islets from 3 separate perifusions for each condition.

**Figure 6. Exendin-(9-39) did not impact $[Ca^{2+}]_i$ in $SUR^{-1/2^-}$ islets.** Isolated $SUR^{-1/2^-}$ mouse islets were cultured with 10 mM glucose for 3 days on coverslips. $[Ca^{2+}]_i$ was continuously measured by Fura-2 fluorescence in response to amino acids (4 mM) in the presence (gray line) or absence of exendin-(9-39)
(black line). Representative experiments are shown. All studies were repeated at least 3 times and showed comparable results.

Figure 7. **Proposed mechanism of action of exendin-(9-39) in SUR-1<sup>-/-</sup> islets.** In SUR-1<sup>-/-</sup> mouse islets, plasma membrane depolarization results in elevated cytosolic Ca<sup>2+</sup> and dysregulated insulin secretion. Exendin-(9-39) binds to the GLP-1 receptor and lowers baseline cAMP levels, resulting in decreased insulin secretion despite the elevated calcium levels. Similarly, by decreasing amino acid-stimulated cAMP accumulation, exendin-(9-39) inhibits amino acid-stimulated insulin secretion.
<table>
<thead>
<tr>
<th>Condition</th>
<th>cAMP Content (pmol/100 islets)</th>
<th>Insulin Secretion (ng/100 islets/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (n=3)</td>
<td>40±4</td>
<td>221±22</td>
</tr>
<tr>
<td>100 nM Exendin-(9-39) (n=3)</td>
<td>21±2a</td>
<td>126±17a</td>
</tr>
<tr>
<td>4 mM AAM (n=8)</td>
<td>73±13ac</td>
<td>360±32bd</td>
</tr>
<tr>
<td>100 nM Exendin-(9-39)/4 mM AAM (n=4)</td>
<td>24±5a</td>
<td>190±35</td>
</tr>
</tbody>
</table>

Table 1. Exendin-(9-39) reduces baseline and stimulated cytosolic cAMP content and insulin release in SUR-1-/- islets. Isolated SUR-1-/- mouse islets were cultured in 10 mM glucose for 3 days. Islets were preincubated in glucose free KRBB for 60 min. 100 nM Exendin-(9-39) was added after 30 min preincubation. Then islets were exposed to different treatment conditions for an additional 30 min. All conditions contained 0.1 mM IBMX. Compared with baseline condition, a: p<0.05, b: p<0.01. Compared with 100 nM Exendin-9/4 mM AAM, c: p<0.05, d: p<0.01.
<table>
<thead>
<tr>
<th>Condition</th>
<th>cAMP Content (pmol/100 islets)</th>
<th>Insulin Secretion (ng/100 islets/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline, G 0</td>
<td>83±3</td>
<td>17±4</td>
</tr>
<tr>
<td>100 nM Exendin-(9-39)</td>
<td>47±6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16±6</td>
</tr>
<tr>
<td>10 mM Glucose</td>
<td>62±4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>153±10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 mM Glucose/100 nM Exendin-(9-39)</td>
<td>44±13</td>
<td>72±11&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>10 mM Glucose/4 mM AAM</td>
<td>95±6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>463±27&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>10 mM Glucose/4 mM AAM/100 nM Exendin-(9-39)</td>
<td>42±2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>153±18&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 2. Exendin-(9-39) reduces glucose and amino acid-stimulated cytosolic cAMP content and insulin release in wild-type islets. Isolated wild-type islets were cultured in 10 mM glucose for 3 days. Islets were preincubated in glucose free KRBB for 60 min. 100 nM Exendin-(9-39) was added after 30 min preincubation. Then islets were exposed to different treatment conditions for an additional 30 min. All conditions contained 0.1 mM IBMX. Compared to G 0, a: p<0.01, b: p<0.05; compared to G10, c: p<0.05, d: p<0.01; compared to G10/AAM 4, e: p<0.01.
Figure 1

A. Blood glucose levels (mg/dL) for WT and SUR-1-/− genotypes with a p-value of 0.00000003.

B. Body weight (g) comparison between WT and SUR-1-/− genotypes.

C. Blood glucose (mg/dL) over time (min) showing a significant increase for WT compared to SUR-1-/− with an ANOVA p-value of <0.0001.

D. Insulin levels (pM) over time (min) with a significant difference between WT and SUR-1-/− genotypes, p=0.02, ANOVA.
Figure 2

The diagram illustrates blood glucose levels (mg/dL) across different groups: WT, WTEx9, SUR-1-/-, and SUR-1-/-Ex9. The statistical significance is indicated by p-values: p=0.000002 and p=0.0006.
Figure 3
Figure 4

(A) Graph showing blood glucose levels (mg/dL) over time (min) for different genotypes: WT, WTEx-9, SUR-1-/-, and SUR-1-/-Ex-9. The y-axis represents blood glucose levels, and the x-axis represents time in minutes.

(B) Graph showing Time (min) for the same genotypes as in (A).

(C) Graph showing % Baseline Blood Glucose over time (min) for the same genotypes as in (A).
Figure 5A

Insulin Release (ng/100 islets/min) vs. Duration of Perifusion (min)

AAM Ramp
(0 to 12 mM)

Exendin-(9-39) 0 or 100 nM

KCl
AAM ramp
(0 to 12 mM)

G, 10 mM
± Exendin-9 100 nM

Figure 5B
Figure 7

Insulin secretion

Depolarization

ATP-sensitive K⁺ channel

Voltage-dependent Ca²⁺ channel

Amino acids

cAMP

Ca²⁺

Insulin secretion
Exendin-(9-39) corrects fasting hypoglycemia in sur-1- mice by lowering cAMP in pancreatic β-cells and inhibiting insulin secretion
Diva D. De León, Changhong Li, Madeleine I. Delson, Franz M. Matschinsky, Charles A. Stanley and Doris A. Stoffers

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