Glucose Regulates the Release of Orexin-A From the Endocrine Pancreas

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Orexins (hypocretins) are novel neuropeptides that appear to play a role in the regulation of energy balances. Orexin-A (OX-A) increases food intake in rodents, and fasting activates OXA neurons in both the lateral hypothalamic area and gut. OXA is also found in the endocrine pancreas; however, little is known about its release or functional significance. In this study, we show that depolarizing stimuli evoke the release of OXA from rat pancreatic islets in a calcium-dependent manner. Moreover, OXA release is stimulated by low glucose (2.8 mmol/l), similar to glucagon secretion, and inhibited by high glucose (16.7 mmol/l). Fasting increases plasma OXA, supporting the idea that orexin is released in response to hypoglycemia. Cells that secrete glucagon and insulin contain OXA and both cell types express orexin receptors. OXA increases glucagon secretion and decreases glucose-stimulated insulin release from isolated islets. OXA infusion increases plasma glucagon and glucose levels and decreases plasma insulin in fasted rats. We conclude that orexin-containing islet cells, like those in the brain and gut, are glucosensitive and part of a network of glucose “sensing” cells that becomes activated when blood glucose levels fall. OXA may modulate islet hormone secretion to maintain blood glucose levels during fasting. Diabetes 52: 111–117, 2003

Orexins (hypocretins) are novel neuropeptides that play a role in arousal and the regulation of energy balances (1–3). Orexin-A (OX-A) and -B are 33- and 28-residue peptides, respectively, that are isolated from the rat hypothalamus and named for their ability to stimulate feeding when injected into the brain (2). Their common precursor, prepro-orexin, is expressed in a specific population of neurons in and around the lateral hypothalamic area (LHA) (2), a region classically implicated in the control of mammalian feeding behavior (4). The LHA contains glucosensitive neurons that are stimulated by falls in circulating glucose (5) and inhibited by prandial signals, such as the presence of food in the gut and/or a rise in portal glucose concentration (4). Previous studies have suggested that orexin neurons might correspond to this neuronal population, since orexin neurons are activated by hypoglycemia (6) and increased hypothalamic prepro-orexin mRNA levels are observed in response to fasting (1,7); however, glucosensitive neurons do not contain OXA (8). They are excited by the peptide (8), implying that orexin acts as a neuromodulator at glucosensitive cells, stimulating them under conditions of hunger.

Glucosensitive neurons are also found in regions outside the hypothalamus, including the nucleus of the solitary tract (NTS) (9) and enteric nervous system (ENS) (10). The NTS relays visceral afferent signals to the LHA (8). Orexin fibers are distributed extensively in the NTS (11), and glucosensitive neurons are activated by the same conditions that stimulate orexin neurons in the LHA (6). The ENS participates in the regulation of food intake by directly sensing, integrating, and regulating the machinery of the gut involved in energy metabolism (3). We have previously shown that orexin neurons are located throughout the ENS and, like their hypothalamic counterparts, are activated by fasting (12). Orexins may sense nutritional status in the gut, modulating secretion and motility in order to prime the digestive tract in preparation for food ingestion and energy uptake. Endocrine cells in the gastric and intestinal mucosa (12,13), as well as in the pancreas (12), also contain orexins; therefore, orexins may function as circulating hormones and/or paracrine or autocrine transmitters. The presence of OXA in human plasma supports this idea (14,15).

Little is known about the regulation and role of the orexins in the endocrine pancreas. We have previously shown that OXA and orexin receptor immunoreactivity is displayed by the insulin-secreting β-cells in the rat pancreas (12). OXA release in the extracellular medium might allow β-cells to regulate neighboring cells that express orexin receptors. The present study was conducted to determine whether 1) endogenous OXA is secreted from rat pancreatic islets, 2) OXA release is modulated by extracellular glucose concentrations, and 3) OXA affects pancreatic hormone secretion.

RESEARCH DESIGN AND METHODS

Animals. Adult female or male Sprague-Dawley rats (150–300 g) were used. Animals were killed by CO2 inhalation and exsanguinated. The Animal Care and Use Committee of the State University of New York Downstate Medical Center and the local ethics committee for animal experimentation in Stockholm, Sweden, approved the experimental protocols.

Isolation and culture of pancreatic islets. Pancreatic islets were isolated by digestion with collagenase as previously described (16). Islets were

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EIA, enzyme immunoassay; ENS, enteric nervous system; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; IR-OXA, immunoreactive Orexin-A; LHA, lateral hypothalamic area; NTS, nucleus of the solitary tract; OXA, Orexin-A; OXR1, orexin receptor 1; TFA, trifluoroacetic acid; TTX, tetrodotoxin.
selected under a dissecting microscope, transferred to Petri dishes, and cultured for 20–22 h in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10 mmol/l glucose.

**Static incubation of pancreatic islets.** Incubation of pancreatic islets was performed as previously described (16,17). Briefly, groups of 10 islets were preincubated in 1 ml of Krebs bicarbonate-buffered solution (115 mmol/l NaCl, 5 mmol/l KCl, 2.56 mmol/l CaCl₂, 1 mmol/l MgCl₂, and 24 mmol/l NaHCO₃) containing 0.5% (wt/vol) albumin (fraction V, radioimmunoassay grade; Sigma Chemical, St. Louis, MO) and glucose were studied. In experiments with tetrodotoxin (TTX), diazoxide, and nifedipine (Sigma Chemical), the test substances were present in the medium during both the prestimulation and stimulation incubations. The effects of high KCl, veratridine (Sigma Chemical), OXA (Peninsula Laboratories, San Carlos, CA), and glucose were studied. In experiments with tetrodotoxin, the medium was aspirated and stored at 4 °C in a shaking water bath. At the end of the test incubations, the medium was aspirated and stored at 20 °C. The effects of high KCl, veratridine (Sigma Chemical), OXA (Peninsula Laboratories, San Carlos, CA), and glucose were studied. In experiments with tetrodotoxin (TTX), diazoxide, and nifedipine (Sigma Chemical), the test substances were present in the medium during both the prestimulation and stimulation incubations.

**OXA extraction and measurement.** Samples were loaded onto a Sep-Pak C₁₈ cartridge (Peninsula Laboratories), which was pretreated with acetoni-diochloroacetic acid (TFA). After washing with TFA, adsorbed peptides in the cartridge were eluted with 60% CH₃CN/0.1% TFA. The eluate was dried under vacuum in a Speed Vac SC100. Then, the dried extract was redissolved in methanol, and 10 µl was loaded onto a C₁₈ Sep-Pak cartridge (Peninsula Laboratories). The column was eluted with a linear gradient of CH₃CN containing 0.1% TFA at a ow rate of 30 µl/min. Fractions (0.7 ml) were collected continuously. Each fraction was dried, reconstituted with assay buffer, and assayed.

**Blood sampling.** Blood was collected in propylene tubes containing EDTA (1 mg/ml blood) and aprotinin (500 KIU/ml), centrifuged, and then stored at −80 °C. Plasma-immunoreactive OXA, insulin, and glucagon levels were measured in triplicate. Blood glucose was determined by the glucose oxidase method using a glucose analyzer.

**Glucagon and insulin measurement.** Glucagon was measured using a rat glucagon EIA kit (Peninsula Labs) and human glucagon as standard. Glucagon concentrations were determined against a known standard (0–25 ng/ml), and the detection limit of the assay was 60–80 pg/ml of sample.

**Immunocytochemistry.** The pancreas was fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4), embedded in OCT (TissueTek), and sectioned (10 µm) using a cryostat-microtome. To locate proteins in the tissue by immunocytochemistry, preparations were exposed to PBS containing 1% Triton X-100 and 4% horse serum for 30 min. Primary antibodies were against OXA (affinity-purified rabbit polyclonal, diluted 1:500; Alpha Diagnostic Inter-
Differences were considered significant at \( P < 0.05 \) vs. control.

RESULTS

Depolarizing stimuli evoke the release of OXA. In the first part of this study, we characterized the in vitro release of immunoreactive OXA (IR-OXA) from isolated islets in response to various depolarizing stimuli. Basal IR-OXA release in the presence of 2.8 mmol/l glucose was 2.67 ± 0.28 fmol islet\(^{-1} \cdot \text{min}^{-1} \) \((n = 8)\). When characterized by reversed-phase HPLC, islet IR-OXA eluted as a peak that was superimposable with the peak of synthetic OXA (Fig. 1A). Depolarization of the incubated islets with 80 mmol/l KCl induced a 61\% \((n = 8, P < 0.05)\) increase in IR-OXA release over the basal level (Fig. 1B), suggesting that at least some OXA is stored and released via a regulated pathway. The release of IR-OXA was also significantly increased by the alkaloid veratridine, a Na\(^+\) channel activator (Fig. 1C). Veratridine (10 \(\mu\)mol/l) induced a 47\% \((n = 5, P < 0.05)\) increase in the release of IR-OXA over the mean basal level. The effect of veratridine was concentration-dependent and blocked by the Na\(^+\) channel inhibitor TTX (3 \(\mu\)mol/l; 109.43 ± 8.29\% vs. 100 ± 2\% of basal level, \(n = 6\)) (Fig. 1C). IR-OXA release produced by high KCl (80 mmol/l) was completely inhibited by pretreating islets with the \(\text{L-type } \text{Ca}^{2+} \) channel blocker nifedipine (25 \(\mu\)mol/l, \(n = 8\)) (Fig. 1B). Thus, the stimulated release of OXA depends on membrane depolarization and \(\text{Ca}^{2+}\) influx through \(\text{L-type } \text{Ca}^{2+}\) channels.

The release of OXA is regulated by glucose. Next, we examined whether the release of OXA from islets is regulated by extracellular glucose concentrations. An increase in the glucose concentration from 2.8 to 16.7 mmol/l caused a significant decrease in islet IR-OXA release from 100.00 ± 15.67 to 8.20 ± 2.07\% of control \((P < 0.05)\) (Fig. 2A). Insulin release was determined from the same samples that were used to determine OXA release. Insulin secretion increased from 100.00 ± 18.98 to 283.58 ± 35.34\% of control \((n = 8, P < 0.05)\) (Fig. 2A). The finding that IR-OXA release was still reduced in the presence of the ATP-sensitive K\(^+\) channel opener diazoxide (25 and 50 \(\mu\)mol/l) (Fig. 2A) indicates that the inhibitory effect of glucose is not due to insulin- or glucose-induced membrane depolarization.

Conversely, when the glucose concentration was decreased from 16.7 to 2.8 mmol/l, IR-OXA release significantly increased from 100.00 ± 15.95 to 218.96 ± 49.89\% \((P < 0.05)\) (Fig. 2B, top). As expected, glucagon release was also significantly increased (from 100.00 ± 30.17 to 288.27\%, \(P < 0.05\)) (Fig. 2B, bottom). Thus, a negative relationship between glucose concentration and OXA release by pancreatic islets was observed in the present study.

Plasma OXA is increased during fasting. Since the release of OXA is stimulated by low glucose, we hypothesized that the release of OXA is increased during fasting. To test this idea, IR-OXA was measured in the plasma of fasted rats. Plasma IR-OXA eluted as a single peak that was superimposable with the peak of islet release (data not provided).
shown). Food deprivation resulted in a significant ($P < 0.05$) rise in plasma IR-OXA (from $33.1 \pm 6.4$ to $53.4 \pm 11.5$ pg/ml, $n = 6$). These findings are consistent with the hypothesis of increased OXA release during fasting.

**Islet cells display OXA and orexin receptor immunoreactivity.** The increase in release of islet IR-OXA and glucagon by low glucose prompted us to determine whether the same cells produce OXA and glucagon. As
previously reported (12), OXA immunoreactivity was observed in the cytoplasm of β-cells (Fig. 3A); however, intense OXA immunoreactivity was also observed in cells located at the periphery of a subset of islets (Fig. 3B). Double labeling with antibodies to OXA and glucagon revealed that OXA immunoreactivity is displayed by the glucagon-secreting α-cells (Figs. 3C and D). In addition, both β- and α-cells display OXR1 immunoreactivity (12) (Figs. 3E and F), suggesting that OXA might affect insulin and/or glucagon secretion.

OXA modulates glucagon and insulin secretion from isolated islets. We examined the effects of OXA on hormone release from isolated islets. OXA (100 nmol/l) significantly (*P < 0.001) increased glucagon secretion (from 100 ± 17 to 200.74 ± 13.47%, n = 8) from islets incubated in low (2.8 mmol/l) glucose (Fig. 4A) and had no effect on the insulin level (not shown). A significant increase (*P < 0.05) in glucagon secretion was also observed in the presence of 80 nmol/l OXA; however, no effect was observed at concentrations in the range of 1–50 nmol/l (Fig. 4A).

When the glucose concentration was raised to 16.7 mmol/l, OXA (100 nmol/l) significantly (*P < 0.05) decreased glucose-stimulated insulin secretion (605.93 ± 48.26 to 375.38 ± 52.95%, n = 8) (Fig. 4B). A significant (*P < 0.001) decrease in glucose-stimulated insulin secretion was also observed in the presence of 150 nmol/l of OXA (from 252.48 ± 25.66 to 605.93 ± 48.26%) (Fig. 4B); however, no effect was observed at concentrations in the range of 1–80 nmol/l (Fig. 4B).

OXA modulates plasma insulin, glucagon, and glucose in fasted rats. Next, we determined whether OXA modulates plasma insulin, glucagon, and/or glucose level. Like the exogenous application of OXA to islets incubated in low glucose, the intravenous administration of the peptide (100 pmol · kg⁻¹ · min⁻¹ for 20 min) significantly (*P < 0.05) increased plasma glucagon in fasted rats (from 252.48 ± 25.66 to 605.93 ± 48.26%) (Fig. 4B); however, no effect was observed at concentrations in the range of 1–80 nmol/l (Fig. 4B).

FIG. 4. Effect of OXA on glucagon and insulin secretion from isolated pancreatic islets. A: OXA (80 and 100 nmol/l) potentiates the release of glucagon from rat islets in the presence of low glucose (2.8 mmol/l). B: OXA (100 and 150 nmol/l) inhibits glucose (16.7 mmol/l)-stimulated insulin secretion. Results are expressed as percentages of control immunoreactive (IR) glucagon (0.21 ± 0.04 fmol · islet⁻¹ · min⁻¹, n = 8) or insulin (1.96 ± 0.88 fmol · islet⁻¹ · min⁻¹, n = 8) release in the presence of 2.8 mmol/l glucose. *P < 0.05 vs. without drug.

FIG. 5. Effect of OXA on blood glucose levels. Animals were food deprived for 12 h before infusion of vehicle (isotonic saline; ■) or OXA (80 pmol · kg⁻¹ · min⁻¹; □). Glucose concentrations were measured every 20 min. Time 0 corresponds to the beginning of vehicle or OXA infusion. Blood glucose levels are expressed as percentages of mean blood glucose concentration observed at time 0. Values represent means ± SE of five independent experiments. *P < 0.05 vs. control.
plasma insulin concentration significantly ($P < 0.05$) decreased (from $10.99 \pm 0.91$ to $1.97 \pm 0.91$ ng/ml, $n = 4$). Interestingly, plasma glucose concentrations were significantly higher during and after OXA (80 pmol $\cdot$ kg$^{-1}$ $\cdot$ min$^{-1}$) administration (Fig. 5), as might be expected due to increased glucagon and reduced plasma insulin levels.

**DISCUSSION**

We have previously reported that OXA and orexin receptors are expressed in the endocrine pancreas (12); however, the physiological significance of this expression was unclear. Many neuropeptides are contained in granule-like structures within endocrine cells, from which they can be released into the extracellular space. The study of this secretion represents an important step toward the identification of mechanisms involved in the modulation of its function and, ultimately, an understanding of its function. The present study demonstrates that OXA is released from pancreatic islet cells and that glucose levels regulate its release. In addition, it shows that OXA can modulate pancreatic hormone secretion and blood glucose levels.

We characterized the release of OXA from isolated pancreatic islets using an EIA capable of detecting <1 fmol of IR-OXA and confirmed the authenticity of the released peptide by reversed-phase HPLC. As is typical of the release of peptides, depolarizing stimuli produced a dose-dependent increase in the release of OXA that was TTX sensitive. Furthermore, the release of OXA evoked by 80 mmol/l KCl was abolished in the presence of nifedipine; therefore, OXA is stored and released by islet cells, and $L$-type Ca$^{2+}$ channels are involved in the response. OXA release from pancreatic islet cells is regulated by glucose. A decrease in the glucose concentration from 16.7 to 2.8 mmol/l more than doubled OXA release. In contrast, when the glucose concentration was increased from 2.8 to 16.7 mmol/l, OXA release decreased by ~90%. These findings indicate that OXA release is stimulated by decreased glucose levels and suggest that OXA-containing pancreatic islet cells, like those in the brain (18) and gut (12), are glucosensitive and part of a network of glucose-sensing cells that becomes activated when blood glucose levels fall.

Orexin neurons in the LHA are activated by hypoglycemia (6), and blood glucose inhibits prepro-orexin mRNA expression in these cells (1,7). Thus, orexin cells are somehow sensing the animal's nutritional state, and OXA is released in response to nutritional depletion. In support of this idea, we found a significant increase in plasma OXA level in fasted lean rats similar to that reported in humans (14,15). The origin of plasma OXA was not identified; however, in light of the present findings, one potential source is the pancreatic islets. Other peripheral sites of synthesis include neurons and/or endocrine cells in the gut (12), adrenal glands (19), and testis (2,19,20). Furthermore, since cells outside the central nervous system express orexin receptors (12,19), the findings support the existence of a peripheral orexin system and suggest that OXA may function as a hormone and/or neuromodulator. Peripheral plasma OXA may modulate brain regulatory centers, since OXA crosses the blood-brain barrier (21).

In the rat hypothalamus, OXA is colocalized with dynorphin (22) and galanin (23), two other orexigenic peptides, suggesting that this colocalization might have an important physiological role. In the endocrine pancreas of the rat, OXA immunoreactivity is displayed by both the $\alpha$- and $\beta$-cells, which secrete hormones that antagonistically control the balance between glucose storage and consumption and maintain plasma glucose levels within a very narrow range. Glucagon secretion is increased in the fasting state to activate glycogenolysis and gluconeogenesis, whereas insulin is secreted in response to nutrients to promote energy storage. Interestingly, the regulation of OXA release from isolated islets is similar to that of glucagon secretion. For instance, both OXA and glucagon release are suppressed by high glucose levels, stimulated by low glucose, and increased during periods of fasting. Stimulation of OXA release by low glucose is compatible with a role in the regulation of metabolic states, particularly when food availability is reduced to the organism. The resulting increase in orexinergic activity could then lead to increased glucagon secretion, which is essential for the maintenance of plasma glucose levels during fasting.

The expression of orexin receptors in both pancreatic $\alpha$- and $\beta$-cells suggests that locally released OXA may modulate glucagon and/or insulin secretion. In this study, we show that extracellular OXA significantly increases glucagon secretion from isolated islets incubated in low glucose, while it decreases glucose-stimulated insulin release. Furthermore, the effect of OXA on glucagon and insulin release is concentration dependent. The concentration of OXA necessary to see an effect is higher than that of circulating OXA; however, if OXA were released locally within the islets, the islet cells would be exposed to high concentrations of peptide.

Like the application of OXA to islets incubated in the presence of low glucose, the intravenous administration of OXA to fasted rats increased plasma glucagon and decreased insulin levels. Interestingly, there was a rise in blood glucose, suggesting that OXA may play a role in the physiological control of blood glucose. A role in the control of energy metabolism and maintaining homeostasis has been suggested (24). This may serve to stimulate food intake. Also, an effect on plasma insulin has been confirmed by several approaches (25–28); however, a strong link with glucagon secretion and glycemia is suggested by the present findings.

It may be speculated that fasting, which is associated with a decline in blood glucose, induces an increase in plasma OXA levels. OXA facilitates glucagon secretion, while inhibition of insulin release may become important until normoglycemia is restored. When a meal is ingested and blood glucose rises, a decline in OXA is parallel to that of glucagon and inversely related to insulin. Experiments are in progress to further elucidate the role of OXA in the physiological regulation of blood glucose. A complete understanding will require strict temporal parameters that can only be addressed in future in vivo studies.

In conclusion, the present study demonstrates that OXA is released by islet cells and that these cells, like those in the brain and gut, are glucosensitive and part of a network of glucose-sensing cells that becomes activated when blood glucose levels fall. We propose that islet OXA acts to signal the need to conserve energy and restore blood glucose levels by modulating glucagon and insulin secretion.
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